Studies on hepatic lipid metabolism in the oestrogenized male chick (gallus domesticus)

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STUDIES ON HEPATIC LIPID METABOLISM IN THE OESTROGENIZED MALE CHICK (*GALLUS DOMESTICUS*)

by

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Being a thesis submitted for the degree of
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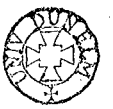

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ABSTRACT OF Ph.D. THESIS

STUDIES ON HEPATIC LIPID METABOLISM IN THE OESTROGENIZED MALE CHICK (*GALLUS DOMESTICUS*)

The aim of this study was to investigate the early lipogenic events occurring in the liver after oestrogen treatment of the male chick *in vivo*.

Liver weight increased with time after an injection of 17β-oestradiol (0·75 mg or 1 mg/100 g body wt.) to at least 40 hours. A dose-related increase in liver weight was observed 48 hours after an injection of 17β-oestradiol over the dose range 0 - 1·25 mg/100 g body weight. Coincident with the increase in liver weight was a decrease in the DNA content and, in many cases, the soluble protein content of a unit weight of liver, substantiating the view that cell expansion, caused by accumulating water and lipid, contributes considerably to the liver enlargement after oestrogen treatment.

Plasma triacylglycerol and phosphoprotein concentrations increased after an injection of 17β-oestradiol (1 mg/100 g body wt.), and became significantly greater than control values at 5 - 7½ and 14 - 18 hours post-injection, respectively.

Liver slice lipogenesis was studied by measuring the incorporation of [1-^{14}C] acetate and $^{3}H_2O$ into total lipid, and of [9,10-^{3}H] palmitate into complex lipids. The incorporation of the radioisotope was predominantly into triacylglycerol in all cases. The livers of oestrogenized male chicks developed a significantly enhanced capacity to synthesize fatty acids *de novo* and to incorporate fatty acids into glycerolipids at times later than 14 hours after hormone injection. Results indicated that a stimulation of *de novo* lipogenesis at early times after injection (≦ 17 h) may be caused by handling and/or the injection of the vehicle propane-1,2-diol.

Treatment of chicks with an optimum dose of 17β-oestradiol (0·75 mg/100 g body wt.) yielded results which suggested that early increases in hepatic fatty acid synthetase activity (≦ 26 h) are caused by handling and/or the injection of propane-1,2-diol, and that oestrogen-dependent changes occur within 48 hours.

CONTENTS

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SYNOPSIS

The aim of this study was to investigate the early lipogenic events occurring in the liver after oestrogen treatment of the male chick *in vivo*.

Liver weight increased with time after an injection of 17β-oestradiol (0·75 mg or 1 mg/100 g body wt.) to at least 40 hours. A dose-related increase in liver weight was observed 48 hours after an injection of 17β-oestradiol over the dose range 0 - 1·25 mg/100 g body weight. Coincident with the increase in liver weight was a decrease in the DNA content and, in many cases, the soluble protein content of a unit weight of liver, substantiating the view that cell expansion, caused by accumulating water and lipid, contributes considerably to the liver enlargement after oestrogen treatment.

Plasma triacylglycerol and phosphoprotein concentrations increased after an injection of 17β-oestradiol (1 mg/100 g body wt.), and became significantly greater than control values at 5 - $7\frac{1}{2}$ and 14 - 18 hours post-injection respectively.

Liver slice lipogenesis was studied by measuring the incorporation of [1-^{14}C] acetate and $^{3}H_2O$ into total lipid, and of [9,10-^{3}H] palmitate into complex lipids. The incorporation of the radioisotope was predominantly into triacylglycerol in all cases. The livers of oestrogenized male chicks developed a significantly enhanced capacity to synthesize fatty acids *de novo* and to incorporate fatty acids into glycerolipids at times later than 14 hours after hormone injection. Results indicated that a stimulation of *de novo* lipogenesis at early times after injection (≤ 17 h) may be caused by handling and/or the injection of the vehicle propane-1,2-diol.

Treatment of chicks with an optimum dose of 17β-oestradiol (0·75 mg/

100 g body wt.) yielded results which suggested that early increases in hepatic fatty acid synthetase activity (≤ 26 h) are caused by handling and/or the injection of propane-1,2-diol, and that oestrogen-dependent changes occur within 48 hours.

GLOSSARY

The abbreviations suggested by the Biochemical Journal (Policy of the Journal and Instructions to Authors; (1984) Biochem. J. 217, 1 - 26) have been adopted. Other abbreviations are as follows:-

G.P.R.	general purpose reagent
VLDL	very low density lipoprotein(s)
LDL	low density lipoprotein(s)
HDL	high density lipoprotein(s)
FLKS	fatty liver and kidney syndrome
FLHS	fatty liver-haemorrhagic syndrome
PHLA	post-heparin lipolytic activity
mRNA	messenger RNA
tRNA	transfer RNA
rRNA	ribosomal RNA
TCA	trichloroacetic acid
DHAP	dihydroxyacetone phosphate
PPO	2,5-diphenyloxazole
PoPoP	1,4-di(2-(5-phenyloxazolyl))benzene
BSA	bovine serum albumin
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
PFS	particle-free supernatant
$r_{av.}$	average radius of rotation

MATERIALS

All reagents were of analytical grade unless stated otherwise. Materials were obtained from the following sources:-

Amersham International plc, White Lion Rd., Amersham, Bucks., U.K.

[1-^{14}C] Acetic acid, sodium salt
[9,10(n)-^{3}H] Palmitic acid

BDH Chemicals, Poole, Dorset, U.K.

Acetaldehyde (G.P.R.)
Ammonium molybdate
Calcium chloride (1 M solution)
Diethyl ether
Ethylenediaminetetraacetic acid (disodium salt)
D-Glucose
Hydrochloric acid
Kieselgel 60H (Merck)
Magnesium sulphate
Orthophosphoric acid
Perchloric acid
Potassium chloride
Potassium dihydrogen orthophosphate
Potassium hydrogen carbonate
Potassium hydroxide
Sodium chloride
Sodium dihydrogen orthophosphate
Sodium hydrogen carbonate
Sodium hydroxide
Sulphuric acid
Tri-sodium citrate

Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K.

Citrate synthase
Dithiothreitol

B.O.C. Ltd., Vigo Lane, Birtley, Co. Durham, U.K.

Nitrogen

Oxygen:carbon dioxide (95%:5%)

J. Burrough Ltd., Fine Alcohols Division, 60. Montford Place, London, U.K.

Absolute alcohol

Fisons Scientific Apparatus, Loughborough, Leics., U.K.

Acetic acid, glacial

Acetic anhydride

Acetone

Chloroform

Cupric sulphate

2,5-Diphenyloxazole (PPO)

Iodine

Isopropanol

Light petroleum (b.p. 40 - 60°C)

Methanol

Potassium iodide

Potassium sodium tartrate

Sodium acetate

Trichloroacetic acid

Triton X-100

Xylene

Gillette Industries Ltd., Surgical Division, Great West Rd., Isleworth, Middlesex, U.K.

SGL 3-blade valet strip razor blades

Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

1,4-Di(2-(5-phenyloxazolyl))benzene (PoPoP)

New England Nuclear, 2. New Rd., Southampton, U.K.

Water, tritium labelled, 100 mCi/g

Sarstedt Ltd., Leicester, U.K.

Semi-micro polystyrene cuvettes (1·6 ml cap.; 1 cm light path)

Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Ascorbic acid
Bovine serum albumin (fatty acid poor)
Cholesterol
Coenzyme A (sodium salt)
Deoxyribonucleic acid (sodium salt from calf thymus, Type 1)
Diagnostic Kit and Reagents for Colorimetric Determination of Triacylglycerols (Technical Bulletin No. 405)
Diphenylamine
5,5'-Dithiobis(2-nitrobenzoic acid)
β-D(-)-Fructose
Lipid standards (mono-, di- and triolein)
Malonyl coenzyme A (lithium salt)
β-Nicotinamide adenine dinucleotide phosphate, reduced form (tetrasodium salt)
17β-Oestradiol
Oleic acid
Oxaloacetic acid
Palmitic acid
Propane-1,2-diol
Tris(hydroxymethyl)aminomethane

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

GENERAL INTRODUCTION

1. The site of lipogenesis

Many organs and tissues are able to synthesize fatty acids, but the liver, adipose tissue and lactating mammary gland have been found to be the major sites of lipogenesis in most species [1, 2]. The metabolic pathways of de novo fatty acid synthesis are similar in the different tissues, but the relative importance of these tissues in fatty acid synthesis may vary as a result of differences in hormonal, nutritional and enzymatic control among species. In many cases, the relative roles of the liver and adipose tissue in lipogenesis are based on the assumption that other tissues and organs are not important in this respect. However, studies in the chick have indicated that this assumption may not be justified, since the intestine, skin and skeleton have been shown to contribute substantially to the total fatty acid synthesizing activity [1, 3 - 5].

In most non-lactating domestic mammals and commonly used laboratory rodents, the adipose tissue is considered to be the principal site of de novo fatty acid synthesis [6]. For example, the adipose tissues of the mouse [7, 8] and rat [9, 10] have been shown to contribute at least 50% and sometimes up to 95% of the fatty acids synthesized. Contrary to these observations, some studies have revealed the liver to be more important than adipose tissue in de novo lipogenesis in mice [11 - 13] and rats [14]. Adipose tissue has been shown to account for nearly all fatty acid synthesis in the pig [15 - 17] and guinea-pig [18]. In non-lactating ruminants, both the liver and adipose tissue contribute to fatty acid synthesis, although adipose tissue is regarded as the more important site [2, 19].

In contrast to the situation thought to exist in most mammals, the liver has been shown to be the principal site of lipogenesis in avian species, including the chicken [20 - 22], pigeon [23], duck [24],

turkey [25], Japanese quail [26], starling and white wagtail [27]. The adipose tissue of the chicken is capable of synthesizing fatty acids [28, 29], but the capacity of chicken liver for de novo fatty acid synthesis is much greater than that of chicken adipose tissue [20, 30, 31]. When compared with rat adipose tissue, the adipose tissues of the chicken and pigeon exhibit a very low rate of de novo fatty acid synthesis [29, 32 - 34], although the ability of avian adipose tissue to manufacture the glycerol portion of triacylglycerols is reasonably high [23, 29]. On the assumption that the liver and adipose tissue are the only lipogenic sites, the contribution of the liver of the chicken to de novo fatty acid synthesis has been calculated to be between 90 and 95% [21]. Conversely, the adipose tissue of the pigeon has been implicated as contributing only about 4% of the total fatty acids synthesized [23, 32]. On the other hand, when sites such as the intestine, skeleton and skin are considered, extra-hepatic tissues have been reported to account for more than 50% of the total fatty acids synthesized by the chick [3, 4]. It would therefore appear that, in avian species, adipose tissue is relatively insignificant as a site of de novo fatty acid synthesis, and functions primarily as a depository for fatty acids synthesized elsewhere in the body. In contrast to the situation in most of the mammals that have been studied, the liver has also been considered to be the major site of de novo lipogenesis in man [35 - 39], although some studies have yielded results implicating adipose tissue as an important site [40 - 43].

Obviously, a discrete organ such as the liver lends itself more readily to metabolic investigations than does adipose tissue with its dispersed location. Since the liver of avian species is very active in lipogenesis, birds have been much used in studies of the mechanisms and regulation of this process. Lipogenesis in bird liver has been

shown to respond to dietary changes in much the same way as that in the liver and adipose tissue of the rat [1]. Hence, starvation or feeding a diet rich in fat depresses lipogenesis and associated enzyme activities, whereas refeeding following a fast or feeding a low fat, high carbohydrate diet results in increased lipogenesis and increased lipogenic enzyme activities [1, 6, 44]. In both the chicken and the rat, refeeding after fasting has been shown to result in increased lipogenesis as compared with ad libitum-fed animals [1, 6, 45, 46]. In the rat, this increased lipogenesis is accompanied by an 'overshoot' of lipogenic enzyme activities [1, 6, 47], whereas birds respond differently and the lipogenic enzyme activities recover to normal or slightly elevated levels [1, 32, 45, 46, 48 - 51]. Birds, and in particular the domestic fowl, have been extensively used in studies of this kind as, in spite of the crop which serves as a food storage organ, food passes through the gut rapidly, and changes in lipogenesis can be evoked relatively quickly. Yeh & Leveille [45] investigated the effects of short-term fasting and refeeding in chicks, and observed that 30 minutes after food removal hepatic fatty acid synthesis from acetate or glucose was depressed. After fasting for 2 hours, the rate of fatty acid synthesis was reduced to about 10% of the rate in ad libitum-fed chicks. Similarly, the return of fatty acid synthesis to normal levels was equally as rapid, occurring after 30 to 60 minutes of refeeding.

2. Egg formation

It is now well-established that the production of eggs by female oviparous vertebrates involves dramatic changes in the composition of the blood and in the metabolism of the liver and oviduct. The onset of breeding in the domestic fowl and other oviparous vertebrates is associated, in the female, with alterations in the types and quantities

of lipids and proteins in the blood [52 - 60]. Furthermore, certain plasma proteins of the egg-laying female show a striking similarity to proteins of egg yolk [61 - 65], whilst the uptake of plasma proteins by developing chicken and amphibian oocytes has been demonstrated *in vivo* and *in vitro* [58, 66 - 70]. Changes in the composition of the blood, therefore, are related to the requirements of the developing egg, and the liver is considered to be the major site of synthesis of these components [58, 71 - 74]. It is now generally agreed that, in many oviparous vertebrates, proteins and lipids destined for the egg yolk are synthesized in the liver, secreted into the blood and are carried to the ovary for deposition in the developing oocyte, whilst egg white proteins such as ovalbumin, conalbumin, ovomucoid and lysozyme are synthesized by the oviduct [75 - 78]. Thus, at the approach of lay in the female domestic fowl, the liver secretes large quantities of specific proteins and lipids into the blood and the hen spontaneously develops endogenous hyperlipaemia, making it a particularly useful experimental animal for studies of lipogenesis.

The physiological changes that occur at the approach of lay in females of oviparous vertebrate species are considered to occur under the influence of steroid hormones, notably oestrogen [60, 79], although undoubtedly a complex interplay of endocrine secretions is involved. The importance of oestrogen in the induction of these changes in the mature female has been shown by the fact that if males and immature females of oviparous species are treated with oestrogens, they respond with increases in liver size and changes in blood composition that are similar to those observed in the laying female [56, 58, 59, 80 - 86].

(a) Protein metabolism

Changes in protein metabolism during the process of vitellogenesis have been studied in some detail in the domestic fowl and the African

clawed toad, Xenopus laevis. At sexual maturity, a large amount of a glycolipophosphoprotein [87, 88] is synthesized by the liver of female animals, and is secreted into the blood [79, 80, 89, 90]. This protein, which has been named vitellogenin [81, 91, 92], is associated with high levels of plasma calcium [60, 73, 79, 87, 92], and is the precursor of the egg yolk proteins, phosvitin and lipovitellin [62, 79, 88, 90, 93, 94]. Like many proteins destined for secretion, vitellogenin is synthesized on membrane-bound polysomes, passes into the cisternae of the endoplasmic reticulum, and is transported to the Golgi apparatus before being secreted from the cell. During this passage to the Golgi apparatus, several secretory proteins undergo post-translational modification [95 - 98]. In the case of vitellogenin, the nascent polypeptide is glycosylated, phosphorylated and becomes associated with lipid [99 - 106].

In addition to the changes in vitellogenin production, some of the proteins that are deposited in the developing egg yolk are normally occurring plasma proteins which are made in the liver, and during vitellogenesis the concentrations of these proteins in the plasma are altered. For example, in the domestic fowl, the concentrations of low and very low density lipoproteins [58, 107] and of various vitamin-binding proteins are increased [65], whilst the level of albumin appears to decrease [58], indicating that the stimulation of protein synthesis is specific rather than general in nature.

(b) Lipid metabolism

All plasma lipids, other than free fatty acids, are transported as part of macromolecular complexes called lipoproteins. The classification and nomenclature of these lipoproteins is based primarily on their electrophoretic mobility or on their rate of ultracentrifugal flotation in salt solutions [108, 109, 110]. Based on these criteria, the major vertebrate plasma lipoprotein fractions are (i) chylomicra,

in which the lipid is of dietary origin, and three fractions in which most of the lipid is produced endogenously, namely, (ii) very low density lipoproteins (VLDL), (iii) low density lipoproteins (LDL) and (iv) high density lipoproteins (HDL). A summary of the composition and properties of these lipoprotein classes, as they occur in the plasma of humans, is given in Table 1. All plasma lipoproteins have the same basic structure and consist of a hydrophobic core, comprising mainly triacylglycerol and cholesteryl ester, surrounded by an amphipathic surface layer of phospholipid, cholesterol and specific proteins (apoproteins). As well as maintaining lipoprotein structure, some apoproteins have specific regulatory functions in lipid metabolism. For example, apo C-II activates lipoprotein lipase (EC 3.1.1.34) and apo A-I activates lecithin : cholesterol acyltransferase (EC 2.3.1.43). A major difference in the lipid metabolism of birds and mammals exists in the absorption of exogenous fat. In avian species, exogenous fat is absorbed via the portal system as large VLDL or 'portomicra' [111, 112], rather than as chylomicra (and VLDL) via the lymphatic system as typically occurs in mammals.

Under the influence of endogenous oestrogen at the approach of lay in the female domestic fowl, the liver synthesizes and secretes large amounts of lipoproteins, mainly VLDL, for export to the developing egg [58, 107]. This situation can be mimicked in males and immature females by administration of exogenous oestrogen, leading to hyperlipaemia [58, 81, 82, 113 - 117, 241]. The lipaemia associated with egg-laying in the hen and the oestrogen-induced lipaemia of immature pullets and cockerels are primarily due to increased triacylglycerol levels, occurring predominantly in the very low density fraction of lipoproteins [107, 113 - 116, 118, 119]. The liver is considered to be the major site of synthesis of VLDL [120, 121] and this, together with

TABLE 1

Composition and properties of the major classes of human plasma lipoproteins

	Chylomicra	VLDL	LDL	HDL
Density (g/ml)	< 0·95	0·95 - 1·006	1·019 - 1·063	1·063 - 1·21
Diameter (nm)	75 - 1000	30 - 70	20 - 25	10 - 15
Classification by electrophoresis	Omega	Pre-beta	Beta	Alpha
Components (Typical composition as % of dry wt.)				
Protein	1 - 2	10	25	50
Triacylglycerol	83	50	10	3
Cholesterol and cholesteryl esters	8	22	43	18
Phospholipids	7	18	22	29
Major apoproteins	B	B	B	A-I, A-II
	C-I, C-II, C-III	C-I, C-II, C-III		
		E		
Minor apoproteins	A-I, A-II	A-I, A-II		C-I, C-II, C-III
		D		D
				E

the observation that without a liver the fowl fails to develop hypertriacylglycerolaemia in response to oestrogen [122], indicates that most of the triacylglycerol is of hepatic origin. The normal chicken diet has a very low fat content and most of the lipid required for the synthesis of egg yolk precursors has to be synthesized from carbohydrate. That the hypertriacylglycerolaemia is not dependent on a dietary source has been shown by the fact that the oestrogen-induced lipaemia still occurs even when birds are deprived of food [82], and also by the fact that the 'portomicron' fraction has been shown to contribute only slightly, if at all, to the oestrogen-induced hyperlipaemia in chicks [113].

Much of this newly synthesized lipid, at least initially, is used for membrane synthesis and proliferation of the endoplasmic reticulum and Golgi apparatus [58, 123, 124], but large amounts of lipid are incorporated into the vitellogenin molecule and are also secreted into the blood in the form of lipoproteins for transport to the egg. Differences between species occur as to the relative importance of synthesis of particular lipids, depending primarily on the composition of the lipid reserves stored in the egg. In contrast to birds and some reptiles, the plasma of Xenopus laevis does not become highly lipaemic [60, 80, 81], and large amounts of calcium are not observed in the blood of Xenopus laevis during vitellogenesis since there is no egg shell production in this species [80].

That increased hepatic lipogenesis is a major and specific feature of vitellogenesis in the domestic fowl, and not simply part of a general increase in metabolic rate, has been indicated by the work of Pearce [125] and Pearce & Balnave [126]. Their studies showed that with the onset of laying, or after oestrogen treatment of immature pullets, the specific activities of certain lipogenic enzymes were increased while the specific activities

of a variety of gluconeogenic, glycolytic and amino acid metabolizing enzymes were unchanged.

3. The action of oestrogen

In mature females of oviparous vertebrate species, increases in hepatic lipid and protein production occur constitutively in response to endogenous oestrogens secreted by the ovary. The secretion of oestrogens, and hence vitellogenesis, is controlled by pituitary gonadotrophins, which are regulated by stimulation via neurohormones from the brain (127 - 129). All steroid hormones are thought to initiate their action by a similar process which involves an initial binding of the hormone to a receptor in the cytoplasm of the target cell, and then translocation of the hormone-receptor complex into the nucleus to combine with acceptor sites on the chromatin. The hormone-receptor complex then stimulates the transcription of selected genes, which results in the increased synthesis of specific proteins (130 - 132).

Many organs and tissues are responsive to oestrogen, including the liver and oviduct of oviparous vertebrate species, and the liver, mammary gland and uterus of mammals. Each organ responds differently to oestrogen, indicating that oestrogen stimulates the transcription of different genes in the various organs. The liver of females of oviparous species responds, as described above, by an increase in lipid production and the synthesis of specific proteins including vitellogenin, vitamin-binding proteins and the apoprotein moieties of lipoproteins, in addition to carrying out its normal functions. The oviduct, on the other hand, responds with a tremendous increase in growth and cell differentiation and the production of egg white proteins (75 - 78). The liver of the female viviparous mammal does not produce egg yolk proteins since the nutrients for embryonic development are provided by

a placental mechanism, but responds to oestrogen with the increased synthesis of VLDL [133 - 137]. Oestrogen also influences certain secondary sexual characteristics of oviparous vertebrates, such as changes in plumage colour and feather shape in avian species which exhibit plumage dimorphism [129].

4. Model systems for the study of vitellogenesis

No significant oestrogen exists in male and immature female animals, and consequently the physiological changes that occur in the mature female are not observed. Nevertheless, studies on male and immature female Xenopus laevis [60, 73, 128, 138 - 140] and chickens [81, 82, 113, 115, 116, 118, 141, 142] have shown that, after oestrogen administration, hepatic lipid and protein synthesis increase, and specific lipids and proteins including vitellogenin, VLDL and vitamin-binding proteins appear in the blood. Male animals have been particularly useful experimental animals for studying oestrogen-dependent changes in liver metabolism which are normally associated with the onset of egg-laying in the female, since any oestrogen-dependent changes in metabolism are caused by the exogenous hormone. This situation is not possible to predict absolutely in immature females, in which oestrogen levels and oestrogen-receptor levels may increase gradually during development. There does not appear to be a limit to the age at which the chicken liver will respond to oestrogen, since even the administration of oestrogen to chick embryos results in the production of plasma lipids and egg yolk proteins [143 - 147].

The oestrogenized male chicken and male Xenopus laevis, therefore, represent a situation in which oestrogen is able to induce or amplify the transcription of specific genes, some of which are normally expressed to varying degrees [115, 116, 148 - 150]. This has proved a

valuable experimental system with which to study the early cellular events in the action of a hormone, particularly the role of steroid hormone receptors and the control of transcription and translation (90). Vitellogenesis has also been induced in vitro in cell and organ cultures of male Xenopus laevis liver (151 - 157) and embryonic and young chick liver (158, 159, 161), thus establishing that oestrogen is the sole inducer required for the synthesis of egg yolk proteins, that the action of the hormone upon the liver is direct, and that DNA synthesis does not appear to be essential for the initial stages of induction. In vitro and in vivo studies have shown that the maintenance of the hormonally-induced protein and lipid production depends upon the continuous presence of oestrogen (90). This characteristic of the vitellogenic response to oestrogen distinguishes hormonal induction from embryonic induction in which the inducer is often dispensable after induction. A lag period normally precedes steroid hormone action, the length of the lag period being typical for a given hormone and a given target tissue. The length of the latent period also varies depending on whether the target tissue has been previously primed by the hormone. This effect is observed in the livers of male oviparous vertebrates after oestrogen treatment, since secondary stimulation results in a shorter lag phase before vitellogenin synthesis is detectable, and also an enhanced response as compared with that obtained with primary stimulation (63, 81, 141, 162). A notable difference between the response of the male Xenopus laevis and that of the cockerel is in the time course of the response. After a single injection of oestrogen, a peak in blood vitellogenin content is observed more rapidly in the cockerel than in the toad, in which the response is more extended. In each case, peak hepatic lipid production occurs at an earlier time than the peak in blood vitellogenin content (63, 81, 138), and both the time course and the

magnitude of the response are affected by the hormone dose level [80, 163].

In mature females, the proteins and lipids synthesized and secreted in response to oestrogens are taken up by the ovaries and accumulate in the egg yolk [80, 89], but in oestrogen-treated males, unable to dispose of them in this way, they accumulate in the blood. The accumulation of vitellogenin in the blood of the oestrogenized male is an important advantage in the purification of the protein, which is of particular interest as a complex protein. Its multicomponent nature makes it an ideal protein with which to study post-translational modifications and processing, which include phosphorylation, glycosylation and lipidation in the liver, and proteolytic cleavage in the ovary to yield phosvitin and lipovitellin. The male Xenopus laevis has been particularly useful in the study of vitellogenin since oestrogen administration does not result in marked lipaemia as in the oestrogenized cockerel, thus making purification procedures easier, and also because chronic treatment of Xenopus laevis with oestrogen results in vitellogenin almost totally replacing all the normal serum proteins [60, 88]. Therefore, a great deal of work has been done using birds and amphibia to examine the transcriptional events and protein synthesis resulting from oestrogenization, but rather less appears to be known about the metabolic events underlying the increased membrane biogenesis and lipid synthesis during vitellogenesis.

Although the administration of oestrogen to immature pullets and cockerels brings about changes in the blood and liver lipids which resemble those observed as the hen comes into lay, quantitative differences may nevertheless exist. Balnave [164] has reported an 'over-reaction' of immature pullets to oestrogen treatment, resulting in heavier livers and greater liver and blood levels of fatty acids relative to those of the mature laying hen. Most notably, there was a

substantial increase in the percentage of palmitic acid in the liver and blood fatty acids of oestrogenized pullets, which was not observed in the mature hen. On the other hand, the mature hen has an increased capacity to retain dietary linoleic acid, which is not reproduced by the administration of exogenous oestrogen to immature female birds. It would, therefore, appear that the administration of oestrogen to the immature female bird does not entirely mimic the situation existing in the mature hen. Undoubtedly, the physiological state of the laying hen is not determined solely by oestrogen, and other ovarian and endocrine secretions may exert an influence. It has been shown that androgens* and progestagens may also be involved in the physiological changes associated with sexual maturity in the hen [165, 166], and the egg-laying process itself may exert a significant effect, since lipid is removed from the body when eggs are laid, and there is a continual demand for lipid for deposition in the developing oocyte. No such lipid demand exists in the immature pullet and cockerel after oestrogen treatment, and lipid and egg yolk proteins accumulate in the blood in the absence of an export system such as egg-laying. It would, therefore, seem likely that any similarities in the lipogenesis of the hen approaching lay and of the oestrogen-treated immature female bird or cockerel would exist before the lipids and egg yolk proteins accumulate in the blood of the oestrogen-treated birds to levels greater than those observed in the mature hen.

5. Agricultural and medical implications

The major agricultural application of the oestrogen-treated domestic fowl, which develops fatty liver and hyperlipaemia, is in the elucidation of the metabolic disorders that give rise to hepatic steatosis in the fowl. Two diseases afflicting the domestic fowl in this way are fatty

* See pages 26 and 34

liver and kidney syndrome (FLKS) and fatty liver-haemorrhagic syndrome (FLHS), which affect young chicks and laying hens respectively [167]. Chicks suffering from FLKS have widespread fatty infiltration of the tissues, particularly of the liver and kidneys which become pale and enlarged [168]. In addition, the chicks are hyperlipaemic, having increased levels of free fatty acids and triacylglycerol in the plasma despite a reduction in hepatic lipogenesis [169]. It is generally believed that the accumulation of fat in the tissues is due to a decreased uptake of lipid by adipose tissue as a result of inhibition of lipoprotein lipase [169]. It has been demonstrated that FLKS can be caused by a combination of nutritional factors, including low biotin, protein and fat levels in the diet, leading to an impairment of hepatic gluconeogenesis [170, 171]. Susceptibility to the disease seems to be precipitated by adverse environmental factors such as stress or fasting, and death occurs as a result of hypoglycaemia [172]. FLHS which affects laying females also manifests itself in the accumulation of fat, of which most is triacylglycerol, in the liver, kidneys and abdominal cavity, and tends to be accompanied by high plasma levels of free fatty acids and triacylglycerol and reduced egg production [167]. Death is usually caused by haemorrhage of the liver. Evidence suggests that the steatosis associated with FLHS is the result of increased hepatic lipogenesis, and it seems likely that the syndrome is caused by an excessive intake of carbohydrate, leading to a positive energy balance, and results from an interaction of dietary and environmental factors. Hence, hens that are caged and at a high environmental temperature and which are unable to reduce their food consumption have a greater susceptibility to FLHS. Other aetiological factors that have been implicated have been a dietary deficiency of some kind and high levels of endogenous oestrogen [167, 280]. In this latter respect, the oestrogen-

treated fowl has received obvious attention, and oestrogen treatment has been shown to result in fatty liver and an increased incidence of hepatic haemorrhage in the domestic fowl [173 - 175].

A number of clinical conditions exist in man in which severe hyperlipaemia is a major problem, notably the exogenous lipaemia of uncontrolled diabetes [176], genetically determined hyperlipoproteinaemia [177], and myxoedaema [178]. In addition, several studies have shown that the level of blood lipids is correlated with the incidence of cardiovascular disease in man [179]. In this respect, the domestic fowl has become a very useful experimental animal in the field of medicine during recent years, since it has been shown that the cockerel develops arteriosclerosis after prolonged oestrogen treatment or cholesterol feeding [180], and that the non-laying hen suffering from FLHS may develop aortic arteriosclerosis [181]. In addition, the oestrogen-treated fowl represents a model for use in studies related to the side-effects of oral contraceptive therapy. The reason for this is that the common usage of oral contraceptives containing oestrogen has brought with it the realization that a major side-effect of such treatment is hyperlipaemia, due principally to elevated triacylglycerol levels [133, 182 - 184]. The domestic fowl is a particularly suitable experimental animal for studies designed to elucidate the mechanisms underlying human endogenous hyperlipaemia, not only because it develops a similar hyperlipaemia when oestrogenized, but because the normal plasma levels of triacylglycerol, cholesterol and free fatty acid are similar to those in humans [113]. In addition, the plasma lipoproteins of the domestic fowl have similar lipid compositions to those of man [109, 113, 185].

Women have been shown to have significantly higher levels of plasma HDL at all ages after puberty than men [186, 187]. In addition, women generally have lower levels of plasma VLDL and LDL than men in middle

age [187]. Epidemiological studies have linked low plasma HDL levels and high plasma VLDL and LDL levels with premature coronary arteriosclerosis [188, 189], and in connection with this it is interesting that women appear to have a lower probability of suffering from arteriosclerotic vascular disease [190]. The higher plasma HDL levels in women are attributed to endogenous oestrogen, and oestrogen administration to men and women leads to elevated levels of plasma HDL and VLDL [191, 192]. The similar or slightly lower plasma VLDL and LDL levels observed in women, compared with the levels in men, are thought to be due to the higher progesterone levels in women [192, 193]. After the menopause, women have an increased risk of coronary heart disease, presumably because there is a decrease in the production of ovarian oestrogen which leads to decreased plasma HDL levels [190, 194]. As previously noted, cholesterol feeding induces aortic and coronary arteriosclerosis in cockerels [180], and it has been shown that oestrogen treatment exerts a protective effect against the development of cholesterol-induced coronary arteriosclerosis in chickens, although hyperlipaemia and aortic arteriosclerosis still occur [195, 196]. With regard to this, it is interesting that cholesterol feeding is unable to induce coronary arteriosclerosis in laying hens [197].

Premenopausal women taking oestrogen-containing contraceptive preparations have been shown to have elevated fasting plasma levels of triacylglycerol, phospholipid and cholesterol [133, 182 - 184, 198]. The major increase is in the triacylglycerol fraction, and the increases in plasma lipids are associated with increased amounts of primarily VLDL [133, 184, 192], and also increased levels of HDL [192, 199] and, in some cases, of LDL [133]. Similar increases in plasma lipoprotein levels have been found in postmenopausal women receiving oestrogen replacement therapy [200]. There are indications that the

increased lipoprotein levels in the plasma of oestrogen-treated women, together with long-term use of oestrogen-containing preparations, may put these women at an increased risk of cardiovascular disease. Evidence has accumulated that the incidence of myocardial infarction [201] and cerebral vascular accidents [202] is significantly increased in premenopausal women taking oral contraceptives compared with those not taking such preparations.

Most oral contraceptive preparations are a combination of an oestrogen and a progestagen, and it is the oestrogen component that is considered to be responsible for the hyperlipaemia [183, 190, 203]. On the other hand, the progestagen appears to be either inert or antagonistic in this respect [134, 190, 193, 204 - 206]. The majority of women taking oestrogen-containing contraceptive steroids experience only mild hyperlipaemia which lies within the normal range, and which may revert to the pre-treatment level after short-term oestrogen therapy [133, 183, 184]. The plasma cholesterol concentration may or may not rise depending partly on the levels that exist before treatment [184, 200, 207]. Occasionally, severe hyperlipaemia develops as a result of oestrogen treatment, particularly in women who have a pre-existing endogenous hyperlipaemia [182, 200]. The greater elevations of plasma lipids in these patients undoubtedly puts them at an increased risk of cardiovascular disease. A solution to this problem would be to develop oral contraceptives without the oestrogen component. Contraceptives containing only progesterone have been introduced, and when used by normal women plasma triacylglycerol levels have been shown to remain unchanged or to decrease slightly [208, 209]. However, reports have shown a higher pregnancy rate and a much higher occurrence of abnormal bleeding in patients receiving these preparations than in women using the conventional oestrogen-containing contraceptives [200].

The domestic fowl and humans both respond to oestrogen treatment with an increase in plasma lipid which is predominantly triacylglycerol. The mechanism of the increase in plasma triacylglycerol levels is controversial, although it seems likely that similar mechanisms are involved in the fowl and in humans. That the hypertriacylglycerolaemia is not the result of an input from a dietary source is supported by the observation that an increase in the concentration of chylomicra is not found in the oestrogen-treated human, and that only a minor increase in the level of portomicra may be seen in the oestrogenized chick [113]. The elevated triacylglycerol levels observed in the plasma after oestrogen treatment could be the result of an increased rate of production of triacylglycerol-rich lipoproteins by the liver or of impaired removal of triacylglycerol from the plasma, or a combination of both of these, and the literature is full of reports supporting each of these possibilities. In addition, in humans, increased levels of insulin [183, 210 - 212], growth hormone [213] and cortisol [214] have been associated with the increase in plasma triacylglycerol, implying that the effect of oestrogen on the induction of elevated plasma triacylglycerol levels may be indirect. That the rise in plasma triacylglycerol levels after oral contraceptive treatment may possibly be related to oestrogen-induced changes in insulin and glucose metabolism is indicated by reduced glucose tolerance in normal women receiving oestrogens [212, 215 - 217]. A number of clinical investigations have been carried out which have indicated that the increase in plasma VLDL associated with oestrogen contraceptive therapy is, at least in part, caused by an oestrogen-induced increase in the hepatic syntheses of apoproteins and triacylglycerol [133, 190, 192, 211, 218, 219]. Similarly, studies on the oestrogenized fowl have shown that the oestrogen-induced rise in plasma VLDL levels is accompanied by increased VLDL synthesis by the liver [114 - 119, 206].

The principal mechanism for the removal of triacylglycerol from the plasma is believed to be via the action of lipoprotein lipase in tissues [220]. This enzyme is thought to function at the luminal surface of the endothelial cells of capillaries, and hydrolyses triacylglycerol in lipoproteins to free fatty acids and glycerol. The fatty acids released are then able to penetrate the tissue cells for further metabolism. Interestingly, some or all of these lipoprotein lipases can be released into the blood by the intravenous injection of heparin, and this has proved extremely useful in measuring lipoprotein lipase activity. Several investigators have reported depressed post-heparin lipolytic activity (PHLA) in women receiving oestrogen-containing preparations [183, 198, 211, 221 - 223], and also in the female rat after similar oestrogen treatment [224]. This phenomenon is also observed in women in the last trimester of pregnancy [225], when there are high levels of oestrogen and associated hypertriacylglycerolaemia. Studies of pregnant animals have shown a similar decrease in adipose tissue lipoprotein lipase activity [226]. Evidence has been presented which suggests that endogenous and exogenous triacylglycerols in the blood are cleared by a common, saturable lipoprotein lipase system [227]. It is, therefore, surprising to find that oestrogens depress PHLA, whilst exogenous fat tolerances have been reported to be normal in oestrogen-treated patients [184, 190, 198, 228]. Hazzard *et al.* [228] have offered an explanation for this by suggesting that the decreased PHLA obtained after oestrogen treatment might be due to a form of resistance of the enzyme to release by heparin, rather than to an actual depletion of lipoprotein lipase. If this was the case, no impairment of plasma triacylglycerol clearance would be evident *in vivo*. On the other hand, some studies on the effects of oestrogen-progestagen oral contraceptives in women have revealed accelerated rates of triacylglycerol clearance

occurring concurrently with increased rates of triacylglycerol production [190, 218, 219].

It has been found that progesterone exerts a protective effect against oestrogen-induced hypertriacylglycerolaemia in rats and humans [134, 190, 204, 205, 224], which is generally considered to be caused by an acceleration of the rate of removal of triacylglycerol from the plasma. For example, Kissebah et al. [190] demonstrated that women using progesterone alone showed a decrease in plasma triacylglycerol concentration with increased triacylglycerol clearance and increased PHLA. Similarly, Glueck et al. [204] provided evidence to support the suggestion that progestational agents lower plasma triacylglycerol levels by bringing about an increase in the activity of lipoprotein lipase. Progestagens have also been shown to reduce plasma triacylglycerol levels and increase PHLA in individuals with familial hyperlipoproteinaemia [229]. It is interesting to note that for any given triacylglycerol production rate, women have been shown to have a lower plasma triacylglycerol level than men, implying that women have a greater capacity to clear triacylglycerol from the plasma [230]. It seems likely that this difference in triacylglycerol clearance between men and women exists because of the higher levels of progesterone in women. Progesterone has not been shown to have any significant effect on the lipoprotein lipase of the domestic fowl [206], although PHLA in the plasma of laying turkeys is decreased relative to non-laying birds, and oestrogen treatment of male turkeys causes a similar decrease in PHLA [231]. An interesting point is that phosvitin, one of the egg yolk proteins induced by oestrogen in birds, has been reported to have an inhibitory effect on lipoprotein lipase from humans, turkeys and chickens [231]. The picture emerging from studies related to the use of oestrogen-progestagen oral contraceptives suggests, therefore, that

the oestrogenic component increases triacylglycerol production, whilst the progestational component may increase the efficiency of triacylglycerol clearance from the plasma [190, 218].

6. The purpose of the present study

It seems likely that oestrogen-induced increases in plasma triacylglycerol levels are predominantly the result of increased VLDL synthesis in the liver. However, controversy exists as to the relative significance of *de novo* fatty acid synthesis in the liver and the influx of fatty acids from extra-hepatic sites in the provision of precursors for complex lipid formation in the liver after oestrogen treatment. It is well-established that, in mammals, plasma free fatty acids are important precursors of plasma triacylglycerol [232]. However, it is also well-known that the liver of avian species has a high capacity for *de novo* fatty acid synthesis. During oral contraceptive therapy elevated plasma free fatty acid levels have been observed [182, 233], and at the onset of lay in the domestic fowl the amounts of complex lipid and free fatty acid in the plasma increase simultaneously [57, 234]. A similar increase in plasma triacylglycerol and free fatty acid levels is seen in oestrogenized male and immature female birds [113, 127, 164, 235]. Kudzma *et al.* [113] stated that the increase in plasma free fatty acids did not occur until the hypertriacylglycerolaemia was well-established in oestrogenized chicks, although the significance of this statement is uncertain because a parallel increase in plasma free fatty acids was also observed in control chicks. The data of Pageaux *et al.* [235], who studied oestrogenized 16-day old female quail (0·2 mg oestradiol benzoate/kg body wt.), are interesting since they show a significant decrease in serum free fatty acid levels 1 hour after hormone injection, followed by increased levels over the next 23 hours. Again, the

significance of these results is uncertain, since values were not presented for control birds at equivalent times after injection, and hence it cannot be deduced whether the changes in plasma free fatty acid levels were hormone-induced or were caused by handling and/or injection of the vehicle. Oestrogen treatment of Xenopus laevis has been shown to lead to an increase in unesterified fatty acids in the plasma [60].

Hawkins & Heald [236] have demonstrated that liver slices from the laying hen incorporate more palmitate into neutral lipids than do liver slices from immature female fowl. Similarly, liver slices from oestrogen-treated immature female domestic fowl exhibit an enhanced capacity to incorporate palmitate into neutral lipids [236]. In vivo studies have shown that there is increased incorporation of plasma free fatty acids into triacylglycerol by livers of oestrogenized immature fowl [114]. However, reports of the rate of de novo hepatic lipogenesis from non-lipid precursors have been confusing, with some investigators reporting a lower incorporation of acetate into fatty acid by mature hen liver slices than by growing chick liver slices [33, 237, 238], others reporting similar incorporation rates of acetate into fatty acids by liver slices from hens and chicks [239], whilst others have demonstrated increased levels of incorporation of acetate into fatty acids by liver slices from oestrogenized chicks compared with those of control chicks [113, 238]. It would, therefore, seem possible that fatty acids from extra-hepatic sites, such as adipose tissue, may be partly involved, if only in the initial stages of the response, in the increased production of triacylglycerol by the liver of the hen approaching lay and of oestrogenized individuals. An alternative, but perhaps unlikely explanation for the observed increases in plasma free fatty acids could be that such vast amounts of fatty acids are manufactured by the liver that some are able to 'leak out' unesterified into the blood. In the

long-term at least, it seems likely that the liver does increase its capacity to synthesize fatty acids de novo for incorporation into complex lipids, as well as its capacity to manufacture these complex lipids from exogenous and endogenous fatty acids. The aim of this study was to add to the present knowledge of the early lipogenic events occurring in the liver after oestrogen treatment of the male chick in vivo.

CHAPTER 2

CHANGES IN WEIGHT AND DNA CONTENT OF THE LIVER AND IN PLASMA TRIACYLGLYCEROL AND PHOSPHOPROTEIN LEVELS OF THE MALE CHICK AFTER 17β-OESTRADIOL INJECTION

INTRODUCTION

1. Factors influencing avian liver morphology

(a) Effects of oestrogen

The changes that occur in the gross physiology of the liver and the constituents of the blood of the female domestic fowl approaching lay have been well-documented (53, 55, 57, 58, 240, 241). At this time the liver becomes enlarged by both cell expansion and cell division (58, 125, 164, 241), as the organ synthesizes egg components in addition to continuing with its normal functions. The hepatic parenchymal cells assume the appearance of active secretory cells, characterized by the extensive proliferation of the endoplasmic reticulum and Golgi apparatus (58, 123). These liver cells are responsible for the production of large amounts of lipid and egg yolk proteins, which are se-

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* However, administration of oestrogen plus androgen to 6-week old male chicks has been found to emphasize changes in the liver produced by oestrogen alone, although the additional increases in liver dry matter, total liver lipid and lipid content (expressed as a percentage of liver weight) did not attain statistical significance (165). The changes in liver fatty acid patterns observed after oestrogen treatment became more pronounced after the administration of androgen with oestrogen (165). Balnave & Pearce (166) injected 4-week old pullets with oestrogen and androgen and observed significantly greater liver weights for these birds than for those treated with oestrogen alone at 3 and 6 hours after hormone injection.

testosterone treatment (253).* A number of investigators have administered oestrogens to the domestic fowl and have observed liver

weights up to, and greater than, double those of control birds [31, 58, 166, 174, 236, 244, 254]. An interesting observation has been reported by Balnave [164] which appears to involve an 'over-reaction' of the liver of the immature female domestic fowl to exogenous oestrogen. After treatment of 16-week old female chickens with 5 mg oestradiol dipropionate on alternate days over a 7-day period, the liver weights of these oestrogen-treated birds were similar to those of heavier mature laying hens. Although the domestic fowl has been extremely popular in studies of liver and blood changes during the vitellogenic response following the administration of oestrogens, similar effects are also observed in other species of birds and other egg-laying vertebrate phyla down to, but not including, the cartilaginous fishes [56, 60]. Another avian species on which much work has been done is the Japanese quail, and oestrogen treatment of 3 to 5-month old male Japanese quail (4·36 mg 17 β-oestradiol/100 g body wt.) has been shown to result in a near doubling of liver weight 4 days after hormone administration [250]. After longer periods of time, the increase in liver weight became less, until gradually the liver weight began to approach the control values.

Contrary to the general observation of increased liver weight after oestrogenization of the chicken, oestrogen treatment of 15-day old chick embryos (5 mg oestrogen/bird) has been found to cause an approximately 15% decrease in liver weight after 3 days, compared with control embryo liver weights [255]. Thirteen-day old embryos and newly hatched chicks, that were injected with oestrogen 3 - 4 days beforehand, were found not to have responded to oestrogen treatment with a change in liver weight, whereas chicks oestrogenized at hatching and sacrificed 3 days later had livers that weighed 28% more than the livers of untreated chicks [146, 255].

Several research workers have demonstrated that oestrogen dose

level and duration of hormone treatment significantly affect the liver weight of the domestic fowl. For example, Balnave & Pearce (166) injected 4-week old immature female chickens with 2 mg oestradiol dipropionate on alternate days over a period of 9 days, and observed a time-related increase in liver weight up to 4 days after the commencement of hormone treatment. At 9 days the response was depressed, although liver weights were still higher than control values. Pearce & Balnave (256) treated 4-week old female chickens with a single injection of oestradiol dipropionate, and 2 days later observed increasing liver weights with progressively larger doses of hormone up to 2 mg/bird. With a dose of 4 mg oestradiol dipropionate/bird liver weight decreased but remained higher than the control value. A similar pattern of response was obtained after 8 days of oestrogen treatment, during which time oestradiol dipropionate (0·5 - 4 mg) was administered on alternate days. In addition, at all oestrogen dose levels the liver weights were significantly greater after 8 days than after 2 days of oestrogen treatment. Pearson & Butler (174) also observed a dose-related increase in liver weight in 6 to 7-week old female chickens that received 5 doses of 0·25, 0·5 or 1 mg oestradiol dipropionate/100 g body weight. Injections were given at 3-day intervals and the birds were sacrificed 2 days after the final dose. Similarly, Akiba et al. (249) have reported increases in liver weight which were proportional to oestradiol dose, after implanting silastic tubes filled with oestradiol dipropionate subcutaneously into the necks of 2 to 3-week old male chicks. Different lengths of oestradiol tubes were implanted with release rates of 1 - 15·2 µg oestradiol/day/bird, and chicks were killed and livers weighed 2 - 3 weeks after implantation.

Increases in liver size and weight of the oestrogenized domestic fowl are usually obvious within 24 hours of hormone administration (166,

242), although Balnave & Pearce (166) found no difference between the liver weights of oestrogenized and control birds 12 hours after oestrogen treatment. As the liver enlarges it becomes paler in colour and fatty in appearance, and develops into a softer, more fragile structure (242). The increase in liver size and weight occurring after oestrogenization is considered to be predominantly due to the increase in volume of existing cells as a result of water uptake and the accumulation of lipid, although it has been estimated that a proportion (< 40%) of the parenchymal cells divide (242, 257). Hawkins & Heald (236) measured liver DNA in 11-week old female chickens treated with 2 mg oestradiol benzoate on alternate days for 7 days and which were sacrificed on the 8th day, and found that the DNA content of a unit wet weight of liver was decreased in these birds compared with values from control birds. In addition, these investigators reported that the DNA content of a unit wet weight of liver was approximately 1·4-fold greater for the immature female domestic fowl than for the laying hen. These observations suggest that the process of liver 'growth' is similar in the hen approaching lay and the oestrogenized male and immature female domestic fowl, in that cell expansion contributes considerably to the increase in liver size, although some DNA synthesis and cell division does occur (248). Interestingly, some reports have revealed a lack of hypertrophy and cellular proliferation in the livers of oestrogen-treated amphibia, and in these cases the ratio of liver weight to body weight remained unchanged (258, 259).

After oestrogenization of male and immature female domestic fowl and at the onset of lay in the mature hen, there is an increase in the total lipid content of the liver, particularly of triacylglycerol which is associated predominantly with LDL and VLDL, and this lipid contributes to the increase in liver weight observed at these times (114, 125, 166, 246, 256). The hepatocytes undergo marked changes in cytology, notably

in the proliferation of the endoplasmic reticulum and the Golgi apparatus with an increase in the number of associated ribosomes and mitochondria (58, 123, 124, 160, 242, 260, 261). The cisternae of the endoplasmic reticulum gradually assume a dilated appearance as they become increasingly active in the production of secretory proteins, and become engorged with lipid droplets and nascent lipoproteins (58). Therefore, in the initial stages of the transformation of these cells, much of the lipid and protein synthesized would appear to be used for membrane biosynthesis, to provide the necessary cellular structures for the ensuing production of large quantities of proteins and lipids for export from the cell. During this time, the number of glycogen granules in the hepatic parenchymal cells is drastically reduced (242).

Other oviparous vertebrates respond to endogenous or exogenous oestrogens with similar changes in liver morphology and cytology to those of the domestic fowl. For example, Xenopus laevis has been shown to respond with an increase in liver size (60), with accompanying development of the endoplasmic reticulum and Golgi apparatus, accumulation of membrane-bound ribosomes, and decreased glycogen content (128, 262 - 265). Liver cells of the lizard, Uta stansburiana, respond to oestrogen in a similar way, and fat vacuoles become prominent in the cytoplasm (242). The adipose tissues of this species normally become enlarged before egg-laying, and it has been suggested that, when oestrogen levels rise, fatty acids are released from adipose tissue into the blood, and are carried to the liver where they are temporarily stored before being utilized. Similar fat vacuoles are observed in hepatocytes of oestrogen-treated Xenopus laevis (153) and domestic fowl (160, 266). It is perhaps interesting to note at this point that oestrogen treatment of female rats has been shown to cause a slight increase in liver weight, involving primarily cell expansion with hypertrophy of the endoplasmic reticulum (136, 267).

(b) Effects of prolactin

Certain other circumstances, apart from oestrogen treatment, have been found in which the avian liver responds with a change in weight. One of the best-documented is the liver enlargement and concomitant stimulation of hepatic lipogenesis associated with the action of prolactin. Prolactin is a pituitary hormone which appears to play an important role in bringing about the metabolic changes necessary for the laying down of fat reserves in migratory birds. Goodridge & Ball [268, 269] studied the response of the pigeon liver to prolactin, and observed that after a daily dose of 1 mg of a prolactin preparation for 5 days the liver had nearly doubled in mass, although liver weight changes were evident even after 1 day of prolactin treatment. It was suggested that imbibition of water was involved at least in the early stages of the increase in liver size. Prolactin also causes the enlargement of the crop sac of the pigeon and the formation of crop milk. It was found that starvation eliminated the increase in liver size caused by prolactin, but that the crop sac still responded [269]. On the other hand, growth hormone was found to cause the same changes in liver size and metabolism as seen with prolactin, but failed to increase the size of the crop sac [269]. These investigators concluded that prolactin causes hyperphagia, which leads to an increase in body weight together with increases in the weights of specific organs such as the liver, pancreas and intestine. Hyperphagia has been shown to cause an increase in liver weight in avian species, principally as a result of lipid deposition [26, 270, 271]. In this respect, it is of interest to note that increased food consumption has been observed in oestrogen-treated immature male and female chickens [174, 246].

(c) Effects of the pattern of food intake and the composition of the diet

The liver weight of the domestic fowl has been shown to fluctuate according to the pattern of food intake and the composition of the diet. Interestingly, chickens do not develop a fatty liver on fasting to the extent seen in mammals [272 - 275]. Leveille [50] demonstrated that the liver weight of the chick decreased during a 3-day fast, increased to greater than the normal weight on refeeding for 2 days, and returned to the control value on the 3rd day of refeeding. During fasting there was a slight increase in the liver lipid content that amounted to about 10%. The increase in liver weight after refeeding correlated with an increase in liver lipid. Muiruri et al. [46] showed that liver weights were greater in meal-fed chicks than in ad libitum-fed chicks. In addition, 1 hour after allowing meal-fed chicks access to food, there was a significant increase in liver weight, which these workers suggested was primarily the result of increases in water and glycogen content and, to a lesser extent, in lipid content [33, 46]. The stimulation of hepatic lipogenesis by fructose is well-known [236, 276], and Pearce [277] has demonstrated that a diet rich in fructose causes a marked increase in liver weight and liver lipid content in 7 to 8-week old female chicks. In some species, including the domestic fowl, the embryonic and newborn young have fatty livers, the lipid being derived from the yolk on which the embryo is nourished. However, this phenomenon is short-lived and the livers become normal a few days after hatching [278, 279].

(d) Fatty liver and kidney syndrome (FLKS) and fatty liver-haemorrhagic syndrome (FLHS)

FLKS and FLHS are two non-infectious diseases afflicting young chicks and laying hens respectively [167], and have been discussed in Chapter 1. The livers of affected birds are pale and enlarged as a result of fatty infiltration, of which most is triacylglycerol. The

accumulation of lipid in the liver in FLKS occurs despite a reduction in hepatic lipogenesis and appears to be due to reduced uptake of lipid by adipose tissue [169], whereas evidence suggests that the accumulation of lipid in the liver in FLHS is the result of increased hepatic lipogenesis [167]. The internal structure of the liver of a victim of FLHS is disrupted greatly by the increased incidence of fat vacuoles in the cytoplasm of hepatocytes which often leads to rupture of the cells, and by lysis of the reticulin bands around the cells which results in structural weakness in the liver. In addition, there are diffuse capillary haemorrhages, bloodclots, vascular breakdown, and regions of fibrosis and necrosis [167, 280]. In cases of FLHS and occasionally of FLKS, small haemorrhages are found on the periphery of the liver, and in FLHS death is usually caused by haemorrhage from the liver which ruptures the liver capsule.

(e) Instances and causes of fatty livers in other species

Fatty livers have been described in several species, including man, as a result of changes in physiological state, disease, poisoning or dietary defects [275, 279, 280]. For example, a fatty liver is associated with conditions such as pregnancy [281], diabetes mellitus, pernicious anaemia, kwashiorkor, alcoholism and a variety of infections [275,279]. Dietary deficiencies in choline and its precursors and in protein result in a fatty liver, as do many other dietary defects including an imbalance of amino acids or vitamins in the diet, too much dietary fat or cholesterol, excessive food intake, and the early stages of starvation. Several chemical substances also induce the formation of an enlarged fatty liver and these include carbon tetrafluoride, chloroform, ethionine, orotic acid and the rare earth metals [275].

Fatty livers can be the result of a variety of abnormal liver functions including accelerated lipid production and/or decreased lipid

degradation, or decreased apoprotein synthesis for the production of lipoproteins. In some cases the fatty liver is caused by an enhanced synthesis of triacylglycerols in the liver, and in these circumstances the plasma triacylglycerol level is either normal or elevated. On the other hand, decreased fatty acid oxidation and/or a decrease in lipoprotein synthesis can also cause triacylglycerol accumulation in the liver. When lipoprotein production is interfered with the levels of triacylglycerol in the blood will be depressed, and this decrease in lipoprotein production may be the result of impaired lipid synthesis or impaired apoprotein synthesis [275].

2. Effects of oestrogen on extra-hepatic organs

It is well-known that oestrogens exert significant effects on the morphology and metabolic activity of organs other than the liver, notably the oviduct of avian species [31, 166, 236, 246, 256, 282, 283], and the uterus [137, 284 - 288], vagina [288] and mammary gland [289] of mammals. Hawkins & Heald [236] observed an approximately 25-fold increase in the oviduct weight of 11-week old chickens after treating the animals with 2 mg oestradiol benzoate on alternate days for 7 days. Similarly, Aprahamian et al. [31] obtained about a 23-fold increase in oviduct weight in 1-month old chickens after treating each bird with 4 mg oestradiol benzoate/day for 6 days. Other research workers have observed similar increases in oviduct weight after treating immature pullets with oestrogen [166, 246, 256], and in addition, have noticed that treatment with a mixture of oestrogen and testosterone causes a greater increase in oviduct weight than observed with oestrogen alone [166, 246]. This increase in oviduct size is associated with cell expansion and division, is accompanied by an increase in metabolic activity and stimulation of ovalbumin synthesis, and is a prerequisite for the oviduct to respond to progesterone.

The uterus of mammals also responds to oestrogen treatment with an increase in size and metabolic activity (137, 285), and it has been shown that oestrogens dramatically increase the number and size of cells and the water content of the uterus (267, 284, 290). The mammalian vagina responds to oestrogen with an increase in the thickness of the epithelium, as a result of an increase in the rate of proliferation of the cells of the basal layers. The cell layers of the vaginal epithelium increase in number and those on the surface stratify, then cornify and finally desquamate (288). The development of the mammary gland during puberty and pregnancy appears to take place in response to several hormonal stimuli which occur in a prescribed sequence. Oestrogen, together with other hormones, is involved in the ductal growth which occurs during puberty, and lobuloalveolar growth which occurs during pregnancy. Ductal growth involves cellular proliferation giving rise to 'determined' but undifferentiated cells, and during pregnancy the interductal spaces are filled with lobuloalveolar structures as a result of cell replication and differentiation. In pregnancy, the epithelial cells assume a secretory function and the gland becomes highly vascularized in preparation for the production of milk (289).

3. Effects of oestrogen on the protein and lipid metabolism of avian liver

In addition to the increase in liver size and weight, another visible effect of oestrogen treatment in the domestic fowl is the development of a yellow clouding of the plasma. This phenomenon is the result of accumulating lipid, and using the technique of functional hepatectomy, Ranney & Chaikoff (122) demonstrated that the liver was responsible for the development of this lipaemia in the oestrogenized cockerel. The lipid constituting the lipaemia is synthesized in the liver and is not of dietary origin, since the lipaemia still develops if

food is withheld [82]. This oestrogen-induced hyperlipaemia is reminiscent of that observed in the mature hen approaching egg-laying, when the liver assumes the role of synthesizing the lipids and proteins required by the developing egg [57, 236]. The most marked elevations in the levels of lipid and egg yolk proteins in the plasma are observed during the 14 days immediately prior to the onset of laying, when the plasma lipid level can rise to 10 - 14 g/100 ml plasma, compared with 0·2 - 0·5 g/100 ml plasma in the immature female bird [55, 127]. When the first egg is laid, the plasma lipids decrease sharply to a level of 1·5 - 3 g/100 ml [127, 291].

The bulk of the plasma lipids constituting the lipaemia associated with egg-laying or oestrogen treatment of the domestic fowl are triacylglycerols, existing predominantly in the form of VLDL [113 - 115, 117, 118, 236, 242]. VLDL are normal constituents of the blood and emanate from the liver [120, 121]. Several workers have shown that oestrogen treatment of the domestic fowl results in a dramatic increase in the plasma concentration of VLDL, with a lesser increase in LDL and a marked decrease in HDL [117, 118, 292]. All the lipoprotein fractions are enriched in triacylglycerol, whilst the proportion of cholesterol is decreased in all the fractions. The remaining hyperlipaemia is attributable to phospholipids and to a lesser increase in cholesterol. The increase in triacylglycerol production by the liver is accompanied by a parallel increase in the production of the apoproteins of LDL and VLDL [115 - 119, 293]. Several investigators have demonstrated the enhanced production of VLDL by the liver of the oestrogen-treated domestic fowl [114 - 116, 118, 160, 206], and VLDL synthesis has been maintained in primary cell cultures isolated from the livers of oestrogen-treated chicks for at least 48 hours [160]. However, only a small degree of success has been achieved in the <u>in vitro</u> stimulation of VLDL synthesis by oestrogen in liver slices from male chicks [159].

The increase in VLDL apoprotein synthesis is the result of the increased transcription of corresponding apoprotein genes [116, 119]. The apoprotein mRNAs are translated on membrane-bound ribosomes and the nascent proteins become associated with lipid, mainly triacylglycerol, during their passage through the endoplasmic reticulum to the cell surface [294]. This increased VLDL apoprotein synthesis is accompanied by similar increases in the syntheses of other secretory proteins such as vitellogenin, which also has lipid associated with it. In the hen, these proteins are destined for deposition in the egg yolk, but they are also manufactured by male and immature female birds in response to oestrogen.

Xenopus laevis responds to oestrogen treatment in a similar way to the domestic fowl, but this species differs from the domestic fowl in that the plasma remains transparent after oestrogen treatment, giving no indication of enhanced VLDL synthesis [60, 81]. However, an increase in hepatic lipid synthesis, primarily of cholesterol and fatty acids, has been detected in the oestrogenized male and female Xenopus laevis [138 - 140], and the bulk of this lipid is incorporated into intracellular membranes, although some of it is associated with the egg yolk proteins. The peak production of lipid in the oestrogenized female Xenopus laevis occurs about 6 days after a single dose of oestrogen (1 mg 17 β-oestradiol) [138, 139], which precedes the peak in vitellogenin content of the blood at about 9 - 21 days after hormone treatment. Similarly, in the oestrogenized cockerel, the increase in concentration of plasma VLDL after a single oestrogen injection occurs earlier and lasts longer than the increase in vitellogenin content of the blood [81]. However, unlike the peak in plasma lipid in Xenopus laevis, that in the oestrogenized cockerel (25 mg oestradiol/kg body wt.) occurs after 2 - 3 days, and after 5 - 6 days begins to return towards normal levels.

Peak vitellogenin content of cockerel plasma is observed at about $2\frac{1}{2}$ - 4 days after such oestrogen treatment.

In connection with the enhanced lipid synthesis associated with egg-laying in the hen and oestrogen treatment of male and immature female domestic fowl, it has been observed that increased levels of free fatty acids accompany the increased lipid content of the plasma [57, 113, 114, 127]. In laying hens, the highest plasma free fatty acid levels are observed during the 14 days before laying begins, when the concentration can reach 4000 µmoles/litre compared with 250 - 500 µmoles/litre in the immature female bird [57]. Similar increases in plasma free fatty acid levels are obtained when immature female chickens are treated with oestrogen [57]. Other oviparous vertebrate species also respond to endogenous or exogenous oestrogen with an increase in plasma free fatty acids [60].

In mammals, plasma free fatty acids are important precursors for plasma lipids which are manufactured in the liver [232], but contrary to the situation thought to exist in most mammals, the liver is a most active site of *de novo* fatty acid synthesis in the domestic fowl [20 - 22]. Hence, the origin of the free fatty acids that appear in the plasma during the onset of laying and after oestrogen treatment of the domestic fowl is somewhat unclear. These fatty acids may have resulted from the lipolysis of triacylglycerol stores in the adipose tissue or have been derived from the lipolysis of VLDL by lipoprotein lipase in peripheral tissues, or they may have been synthesized *de novo* at extrahepatic sites. The fatty acids may, therefore, be in the process of being transported to the liver for incorporation into complex lipids. Alternatively, they may originate from the liver as a result of overproduction. A detailed knowledge of the time sequence of the increased total lipid content and the free fatty acid content of the plasma of

laying hens and oestrogenized birds is lacking, but elucidation of this might help to provide information about the early events occurring in the elevated production of triacylglycerol. If the increase in total plasma lipids was secondary to the increase in plasma free fatty acids, this might imply that the fatty acids originated from extra-hepatic sources such as adipose tissue and were being utilized in the liver for complex lipid synthesis. On the other hand, if the plasma free fatty acids increased after the hypertriacylglycerolaemia was evident, then this might imply that the liver was the site of origin of these fatty acids.

Studies using liver slices and *in vivo* techniques have demonstrated that the liver of the oestrogenized chick has an enhanced capacity to synthesize lipids from non-lipid precursors [113, 114]. In addition, *in vivo* and *in vitro* studies have also shown that the livers of the laying hen and oestrogenized male or immature female bird have an enhanced capacity to incorporate fatty acids into complex lipids [114, 236, 238]. Kudzma *et al.* [113] reported that the increase in plasma free fatty acids in oestrogenized chicks did not occur until after the hypertriacylglycerolaemia was established, suggesting that the liver was not dependent on fatty acids arriving from extra-hepatic sources via the circulation. However, these workers did not explain a parallel increase in plasma free fatty acid levels in control birds which 'masked' the increase in oestrogenized birds. The results presented by Pageaux *et al.* [235] showed that oestrogen treatment of 16-day old female quail (0·2 mg oestradiol benzoate/kg body wt.) resulted in a decrease in serum free fatty acids 1 hour after hormone administration, followed by increased concentrations over the next 23 hours. This implies that, at least initially, the liver may have derived fatty acids from the circulation.

It is interesting to note that there is a substantial increase in

the percentage of palmitic acid in blood and liver fatty acids in oestrogenized pullets compared with the laying hen, implying that fatty acid metabolism in the oestrogenized immature bird does not completely resemble the situation in the mature hen [164]. In addition to an increase in the percentage of palmitic acid in liver fatty acids, oestrogen treatment of immature pullets causes other changes in the fatty acid composition of the liver. For example, increases have been reported in the percentages of palmitoleic and oleic acids, and decreases in the percentages of stearic and linoleic acids [256, 295]. The increase in the percentage of mono-unsaturated fatty acids may be a reflection of the increased desaturation activity observed after oestrogen treatment [296].

Although it has been demonstrated that the oestrogen-induced hypertriacylglycerolaemia is, at least in part, the result of enhanced hepatic VLDL production and secretion, the possibility exists that decreased disposal of plasma lipids via the lipoprotein lipase system may also contribute. Kudzma *et al.* [114] clearly demonstrated, after the administration of [9,10-^{3}H] palmitate, [1-^{14}C] acetate and [U-^{14}C] glucose *in vivo*, that oestrogen treatment of chicks leads to an enhanced capacity of the liver to incorporate fatty acids into triacylglycerol and to synthesize fatty acids *de novo*. Increased production of plasma VLDL was confirmed by a kinetic study of VLDL metabolism, employing reinjected, endogenously prepared [^{14}C] triacylglycerol-labelled VLDL, which showed that the fractional turnover rate of VLDL in oestrogen-treated birds was less than in untreated birds but that the total turnover rate was much greater. Several studies have reported decreased post-heparin lipolytic activity (PHLA) in women being treated with oestrogens [183, 198, 211, 221 - 223], in the female rat after oestrogen administration [224], and also in pregnant women and animals in which high levels of oestrogen and

hypertriacylglycerolaemia exist [225, 226]. Studies on the turkey have revealed that PHLA is reduced in laying females compared with non-laying birds, and that oestrogen treatment of male turkeys causes a similar decrease in PHLA [231]. However, it has been suggested that the reduced PHLA after oestrogen treatment may be due to a certain amount of resistance of lipoprotein lipase to release from the surfaces of the endothelial cells of capillaries by heparin, and that, in fact, the total lipoprotein lipase activity is not depleted [228].

In addition to the elevated levels of VLDL in the plasma of laying hens and oestrogen-treated male and immature female fowl, increased levels of other normally occurring proteins and of certain 'new' proteins are also observed, notably the glycolipophosphoprotein vitellogenin [81, 297], and several vitamin- and mineral-binding proteins [65, 142, 298 - 304]. Contrary to these increased protein concentrations, it has been shown that the plasma concentration of albumin, a normal plasma protein, is decreased in response to oestrogen in the domestic fowl and other egg-laying vertebrates [58, 81, 116, 119, 242, 305, 306]. It has been suggested that this may be due to a decrease in albumin synthesis after oestrogen treatment [152, 258, 266, 307], possibly as a result of decreased synthesis of plasma albumin mRNA by the liver [118, 153, 307 - 311]. However, other workers have implied that the decrease in plasma albumin concentration after oestrogen treatment may be due to an increase in plasma volume rather than to repression of albumin synthesis [81, 174, 175, 250]. This view is supported by reports of little difference existing between the absolute amounts of serum albumin and albumin mRNA synthesized by oestrogen-treated and control animals [118, 306, 308, 312 - 314].

The induction of vitellogenin synthesis in the oestrogenized male animal is a very interesting phenomenon, since it involves the activation

of genes which are not normally expressed significantly (148, 150). This situation has, therefore, been widely exploited in the study of the early events involved in gene expression. In addition, the oestrogenized male has proved useful in the study of vitellogenin itself, since, unlike the situation in the laying female where vitellogenin and the other egg yolk proteins are removed from the blood and incorporated into the egg, no such export system exists in the male animal and the proteins accumulate to high levels in the blood. The synthesis of vitellogenin in response to oestrogens occurs in all oviparous vertebrate species down to, but not including the cartilaginous fishes, but this protein has been studied in most detail in the domestic fowl and Xenopus laevis. That the liver is the site of vitellogenin synthesis has been confirmed by numerous studies including phosphate incorporation studies (67, 315), liver perfusion studies (243), tissue slice experiments (71, 85, 101), tissue culture experiments (72) and hepatectomy (73, 315). Vitellogenin synthesis has been induced in organ and primary cell monolayer cultures of Xenopus laevis liver by oestrogen in vitro, confirming that the action of oestrogen upon the liver is direct and that its continuous presence is required for the maintenance of vitellogenin synthesis (151 - 157). The in vitro induction of vitellogenin synthesis by oestrogen has also been demonstrated in cell cultures of embryonic chick liver (158, 161), although such attempts have been unsuccessful when using liver cell cultures from older birds (266, 316).

Structure of vitellogenin

Vitellogenin is a complex glycolipophosphoprotein which is the plasma precursor of the egg yolk proteins phosvitin and α- and β-lipovitellin (61, 62, 64, 79, 81, 88, 90, 93, 94, 297). Chicken vitellogenin has a molecular weight of about 450,000 and is composed of

2 subunits, each having a molecular weight of 210,000 - 230,000 [93, 317, 318]. Each vitellogenin subunit is proposed to comprise 2 phosvitin residues and 1 lipovitellin residue [64, 317], and Ca^{2+} ions are thought to play an important role in stabilizing the complex between the vitellogenin subunits in the blood of the domestic fowl. It has been suggested that there are at least 2 forms of phosvitin [93, 319, 320] with molecular weights of 28,000 and 34,000 [64, 93], whilst the molecular weight of the lipovitellins is about 170,000 [64]. It is thought that there are 2 vitellogenins in chicken plasma differing in their phosvitin and lipovitellin compositions [320]. One of the forms of vitellogenin gives rise to α- and β-lipovitellins, whilst the other form gives rise to α-lipovitellin only. In addition, neither form can give rise to both of the yolk phosvitins [320]. In connection with this, at least 2 different species of vitellogenin mRNAs are thought to exist in the domestic fowl, with 1 species predominating during primary stimulation with oestrogen [321, 322]. Moreover, diverse forms of vitellogenin have been recognized in the Japanese quail [323]. It has been demonstrated that there are at least 4 different vitellogenin genes in Xenopus laevis [324, 325], and 4 distinct polypeptide products [348]. The vitellogenins of Xenopus laevis are very similar to those of the chicken [325, 326], but contain only 1 type of lipovitellin which resembles the β-lipovitellin of the chicken [93]. Xenopus vitellogenin also contains the bile pigment biliverdin, which gives it a green colour [327].

In the laying hen, the vitellogenin molecule is split into its phosvitin and lipovitellin components within the oocyte [89]. The major proteins in the egg yolk of the domestic fowl are phosvitin, α- and β-lipovitellins, and α-, β- and γ-livetins [79]. The α-, β- and γ-livetins are normally occurring plasma proteins and have been

identified as plasma albumin, α_2-glycoprotein and γ-globulins respectively [243, 349]. These livetins constitute about half of the egg yolk proteins and are present in the egg yolk in an approximate ratio of 1:2:3. Phosvitin and the α- and β-lipovitellins make up most of the remaining egg yolk proteins and are 'new' proteins produced in response to oestrogen. The lipovitellins contribute about 30% and phosvitin about 7 - 8% to the yolk protein [243].

The phosvitins are glycosylated phosphoproteins, and 56% of their amino acids are serine [79]. Most of these serine residues and also some threonine residues are phosphorylated, which means that about 10% by weight of the phosvitins is phosphorus [64, 328]. The lipovitellins are also phosphoproteins but contain about 20% by weight of lipid [261]. In addition, the lipovitellins are rich in methionine residues and about 4% of the amino acid residues are serine. The α- and β-lipovitellins differ from each other in their phosphate content and in their ability to be dissociated into 2 subunits at alkaline pH [329]. It is not altogether clear whether the presence of multiple forms of phosvitins and lipovitellins is the result of polypeptide heterogeneity or of different degrees of lipidation, glycosylation and phosphorylation.

Characteristics of the induction of vitellogenin synthesis

Vitellogenin is the only phosphoprotein present in significant amounts in the plasma of egg-laying vertebrate species, so determination of the amount of phosphorus covalently bound to plasma proteins represents an estimate of the concentration of vitellogenin [81, 250, 330]. The synthesis of vitellogenin and the time course of the appearance of this complex protein in the blood of the oestrogenized male animal have been studied in detail in the domestic fowl and *Xenopus laevis*. *In vivo* and *in vitro* induction of hepatic vitellogenin synthesis by oestrogens is characterized by a lag period before vitellogenin is detectable.

In vivo studies in the cockerel have shown that after a single oestrogen injection, vitellogenin first becomes evident in the blood from about 3 - 4 hours to about 20 hours later, depending on the sensitivity of the detection assay [141, 162, 163, 331], and increases in concentration to reach a peak after 3 - 4 days [81, 141]. The time course and magnitude of the response are affected by the hormone dose level [163, 265], and the response of the cockerel is more rapid than that of the male *Xenopus laevis*, in which the response is slower and more extended [63, 265].

The lag phase is associated with the induced transcription and subsequent translation on membrane-bound polysomes of mRNAs coding for vitellogenin [163, 331, 332], followed by post-translational modification of the nascent protein in the endoplasmic reticulum and Golgi apparatus prior to its secretion [81, 99 - 106]. It is generally considered that phosphorylation occurs after translation [100, 101, 103, 104, 106], but some workers have suggested that phosphoseryl tRNAs may be involved in the synthesis of vitellogenin [333, 334]. In addition to increased synthesis of mRNA during this time, there is also increased production of rRNA and possibly tRNA [248, 257, 335 - 339]. Conflicting views have been expressed as to whether DNA synthesis is required for the induction of vitellogenin synthesis. Several workers have demonstrated enhanced DNA synthesis in the liver of the domestic fowl after oestrogen treatment [247, 248, 257], whilst Green & Tata [152] have suggested that DNA synthesis and cell division are not required for the initial response of the liver, but may be important for maintenance and amplification of the response. Interestingly, the total DNA content of the livers of oestrogenized pullets has been shown to increase concomitantly with enhanced synthesis of vitellogenin [340].

An interesting characteristic of the response of the livers of cockerels and male *Xenopus laevis* to oestrogen is the 'memory effect'

observed after subsequent oestrogen treatments [81, 94, 100, 141, 163, 247, 341]. The 'memory effect' has a very long life-time and in the cockerel is undiminished even after 50 days [81]. The lag phase before the appearance of vitellogenin in the blood is shortened with each successive injection, and an enhanced production of vitellogenin with successive injections has also been shown. This more rapid and enhanced vitellogenin synthesis has been ascribed to the persistence of the demethylated state of the vitellogenin genes [342, 343], to the more rapid and greater accumulation of vitellogenin mRNA with each successive injection [148, 265, 341, 344], to a difference in the rate at which polysomes engaged in the synthesis of vitellogenin become functional [261], and to the pre-existence in subsequent inductions of initiation or elongation factors [345] and special tRNAs [346]. In Xenopus laevis it has been suggested that the more rapid and efficient expression of the vitellogenin genes during secondary oestrogen stimulation may be due to an alteration in the nuclear-cytoplasmic distribution of oestrogen receptor [265, 347]. In addition, there may not be as great a necessity to synthesize endoplasmic reticulum and ribosomes for production of the protein during secondary oestrogen stimulation, as these structures may persist in the cell for some time after primary stimulation.

Interestingly, the 'memory effect' has been found to be virtually absent in the duck [350], and although it is present in the oestrogenized male Japanese quail it has been demonstrated that when male quail have been injected with successive injections of oestrogen (4·36 mg 17 β-oestradiol/100 g body wt./dose) an approximately equal amount of vitellogenin was synthesized each time [250]. Gibbins & Robinson suggested that their lack of enhancement of vitellogenin production with subsequent injections was because they had used a dose of oestrogen that stimulated vitellogenesis maximally in the quail, whilst

they suggested that other workers had used doses that caused sub-maximal stimulation of vitellogenesis in cockerels, and so successive injections were found to lead to cumulative effects [247].

In the following account details are presented of changes in liver weight, liver DNA content, plasma triacylglycerol levels and protein-bound phosphate levels of the plasma at various times after 17β-oestradiol injection of male chicks. These observations were recorded primarily to ascertain that animals had responded to oestrogen treatment. The time courses of changes in these parameters were determined, so as to correlate these changes with the results of later investigations designed to elucidate possible mechanisms involved in the oestrogen-induced hypertriacylglycerolaemia.

METHODS

1. Animals

Day old male Hi-Sex* chicks were obtained from a commercial hatchery and were reared to 2 - 6 weeks of age. The young birds were kept in a communal pen under constant illumination. From 2½ weeks of age, and throughout all experiments, birds were housed in groups of 3 - 6 in cages with raised wire floors. The environmental temperature was 19 - 21°C and a fixed photoperiod of artificial light was maintained. At the beginning of the study the photoperiod was 12 hours/day (7 am → 7 pm), but under these conditions aggression became a problem. To reduce aggression, the photoperiod was decreased to 8 hours/day (9 am → 5 pm) and dimmed red lighting was introduced in all reported work.

Birds were fed ad libitum on standard chick crumbs (Dalgety Spillers, Gold-Start Crumbs ACS**) and were allowed free access to food

* strain-cross White Leghorn ** see Appendix

and water until death. Whenever possible, birds were killed, by decapitation, around midday in order to minimize differences caused by diurnal effects and to ensure, as far as possible, a constant prandial state.

Oestrogen-treated chicks were injected intramuscularly (pectoral muscle) with varying doses of 17 β-oestradiol dissolved in propane-1,2-diol.* Control chicks received an equivalent volume of propane-1,2-diol only.* The inclusion of control, sham-injected birds was considered an important part of all experiments because of possible effects of handling and injection of propane-1,2-diol on metabolism. Goodridge (351) has described a significant stress response in sham-injected control chicks when compared with untreated controls, and lack of appropriate controls in experimental work can lead to speculative and perhaps incorrect conclusions (235).

A variety of plant oils have been used as the injection vehicles for oestrogens by a number of workers (114, 139, 231, 235, 256, 295). The possible effects of these oils on lipid metabolism, even if only in the early stages following injection, favoured the use of non-lipid propane-1,2-diol as solvent in the present study (119, 140, 296, 344), particularly since the early events following oestrogenization were of major interest.

All birds were weighed prior to sacrifice, and immediately after death livers were rapidly removed and weighed before proceeding with experiments. Portions of liver were weighed and stored frozen for future DNA estimation. Blood was collected from the severed necks of birds, and was mixed with tri-sodium citrate to prevent clotting (0·5 ml ice-cold 30% (w/v) tri-sodium citrate/3½ - 4 ml blood collection). Plasma was collected by low-speed centrifugation at 4°C, and plasma samples were stored frozen for future triacylglycerol and protein-bound phosphate analyses.

* 0·1 ml propane-1,2-diol / 100 g body weight

2. Determination of DNA

Liver samples were homogenized in 0·9% (w/v) NaCl [236]. For each gram of liver, 10 ml of 0·9% (w/v) NaCl were added. From duplicate samples of this homogenate the DNA was precipitated, washed and extracted according to the procedure of Prashad & Cutler [352]. The extracted DNA was assayed by the method of Burton [353] with slight modification. Acetaldehyde was omitted from the diphenylamine reagent and was added to the final assay as 0·2 ml of an aqueous solution (1·6 mg/ml) [354]. A 2 ml aliquot of the DNA extract was mixed with 4 ml diphenylamine reagent and 0·2 ml aqueous acetaldehyde (1·6 mg/ml). Incubation was for 18 hours at 30°C, after which the absorbance at 600 nm was measured in a Pye Unicam SP8-100 UV-vis spectrophotometer. Known amounts of standard DNA, and a blank containing 0·5 M-$HClO_4$ only, were treated in the same way. A calf thymus DNA preparation was used as standard. A stock solution of DNA was prepared by dissolving DNA in 0·5 M-$HClO_4$ to a concentration of 0·2 mg/ml. The DNA was solubilized by heating at 70°C for 20 - 30 minutes, and standards of a variety of concentrations (0·025 - 0·2 mg/ml) were prepared by dilution with 0·5 M-$HClO_4$.

3. Assay of plasma triacylglycerols

Triacylglycerol levels in plasma samples were determined using the Sigma Diagnostic Kit procedure described in the Sigma Technical Bulletin No. 405 [355]. The principle of the assay involves the extraction of triacylglycerols from plasma or serum samples into isopropanol, with removal of interfering substances by a solid adsorbant. The triacylglycerols are then subjected to the following reactions:-

(a) Triacylglycerol + KOH $\longrightarrow$ Glycerol + Fatty acids

(b) Glycerol + Periodate $\longrightarrow$ Formaldehyde

(c) Formaldehyde + NH_4^+ + Acetylacetone $\longrightarrow$ Diacetyldihydrolutidine

Diacetyldihydrolutidine is yellow and exhibits maximum absorbance at 410 nm. Absorbance readings at 410 nm are proportional to the triacylglycerol concentration.

The following solutions were made up and stored at 4°C:-

(1) 125 mg sodium m-periodate dissolved in 50 ml 2 M-acetic acid

(2) A chromogenic reagent prepared by mixing 2 M-ammonium acetate (20 ml), isopropanol (40 ml) and acetylacetone (0·15 ml). This reagent required ageing to obtain maximum colour in the final reaction and was kept for at least 18 hours before use.

The solutions were replaced each month.

Activated alumina (0·8 ± 0·2 g) was added to screw cap glass vials. Blank and standard assays were included along with duplicate test assays. Blank vials contained 5 ml isopropanol and 0·2 ml distilled water. Standard vials contained 4·8 ml isopropanol, 0·2 ml distilled water and 0·2 ml triolein standard (300 mg triolein/100 ml anhydrous isopropanol). Test vials contained 5 ml isopropanol, and 0·2 ml plasma was added whilst gently swirling the vial contents.

The vials were shaken manually for 5 minutes, and then the contents were centrifuged at low speed at 4°C to sediment the alumina. Aliquots of the clear supernatant (2 ml) were transferred to tubes, and 0·5 ml 1 M-KOH was added to each with mixing. The tubes were incubated at 60°C for 5 minutes and then cooled rapidly. Periodate solution (0·5 ml) was added to each tube with mixing. Exactly 10 minutes after the periodate addition, 3 ml chromogenic reagent were added with mixing. The tubes were capped with aluminium foil and incubated for 30 minutes at 60°C. After rapidly cooling the tubes to room temperature, the absorbance of the solution at 410 nm was measured. Readings were completed within 20 minutes of the end of incubation. A standard curve was prepared using a triolein standard of 300 mg/100 ml isopropanol.

Initially, plasma samples containing elevated triacylglycerol levels were diluted with 0·9% (w/v) NaCl, and 0·2 ml of the resulting diluted plasma was used for assay. However, when results for varying dilutions of a plasma sample were compared, it became apparent that increasing concentrations of plasma did not show corresponding increases in triacylglycerol content, implying that triacylglycerols in the plasma were not being extracted quantitatively. With the enormous dilutions that were often needed when dealing with plasma from oestrogenized chicks, this posed a substantial problem. A more satisfactory approach was employed involving the extraction of total lipids from plasma samples using the method of Bligh & Dyer [356]. Aliquots of the lipid extract were transferred to vials and were evaporated to dryness under a stream of nitrogen. Isopropanol (5 ml), water (0·2 ml) and activated alumina (0·8 ± 0·2 g) were added to each vial, and the assay procedure was continued as described above.

4. Estimation of protein-bound phosphate in plasma

The proteins in duplicate 0·1 ml aliquots of plasma were precipitated with 2 ml ice-cold 10% (w/v) trichloroacetic acid (TCA). After centrifugation at 1550 g for 20 minutes at 4°C, lipid was removed from the protein precipitate by the following procedure [163]. The precipitate was washed with 2 ml ice-cold 10% (w/v) TCA, 2 ml acetone, twice with 2 ml ethanol/ether/chloroform (2:2:1, by vol.), and then again with 2 ml acetone. The phosphoprotein in the plasma was then assayed by measuring the alkali-labile phosphate content of the lipid-free protein precipitate. The protein precipitate was solubilized in 2 ml 1 M-NaOH and was heated at 100°C for 15 minutes. After cooling, the protein was re-precipitated with 2 ml ice-cold 20% (w/v) TCA and collected by centrifugation [100]. The phosphate content of the resulting supernatant was

determined by the inorganic phosphate method of Ames [357]. Plasma samples were also taken through the procedure omitting the heating stage so as to give a measure of the inorganic phosphate content. Potassium dihydrogen orthophosphate was used as standard.

RESULTS

1. Changes in liver weight after oestrogen treatment

The relationships between the body weight and the organ weights of an animal are affected by parameters such as the species, breed, sex and physiological state of the individual, and environmental and nutritional conditions. Nevertheless, it is generally observed that, at least in the normal young growing animal, an increase in body weight is accompanied by increases in internal organ weights. Matsuzawa [358] has extensively documented the changes in body weight and a variety of organ weights, including that of the liver, of growing male and female White Leghorn chicks during the first 20 weeks of life.

The data presented in Fig. 1 illustrate the relationship between liver weight and total body weight for control (injected with propane-1,2-diol) and untreated male Hi-Sex chicks aged 2 - 6 weeks. The regression equation for these data is:-

$$y = 0{\cdot}029x + 2{\cdot}081$$

where y = liver weight (g) and x = total body weight (g).

The correlation coefficient (r) of 0·928 is statistically significant ($P < 0{\cdot}001$), indicating a very high degree of positive correlation between liver weight and body weight in these chicks. In Fig. 2 the data from Fig. 1 have been replotted to show the relationship between liver weight and body weight minus liver weight for control and untreated

FIGURE 1

The relationship between liver weight and total body weight of control and untreated male Hi-Sex chicks

● values for control chicks

▲ values for untreated chicks

Control chicks had received a single intramuscular injection of propane-1,2-diol (0·1 ml/100 g body wt.) at varying times prior to sacrifice.

Regression equation:-

$$y = 0{\cdot}029x + 2{\cdot}081$$

where y = liver weight (g) and x = total body weight (g)

Chicks were aged 2 – 6 weeks.

Separate regression analyses for control and untreated birds showed that there was no significant difference between these groups.

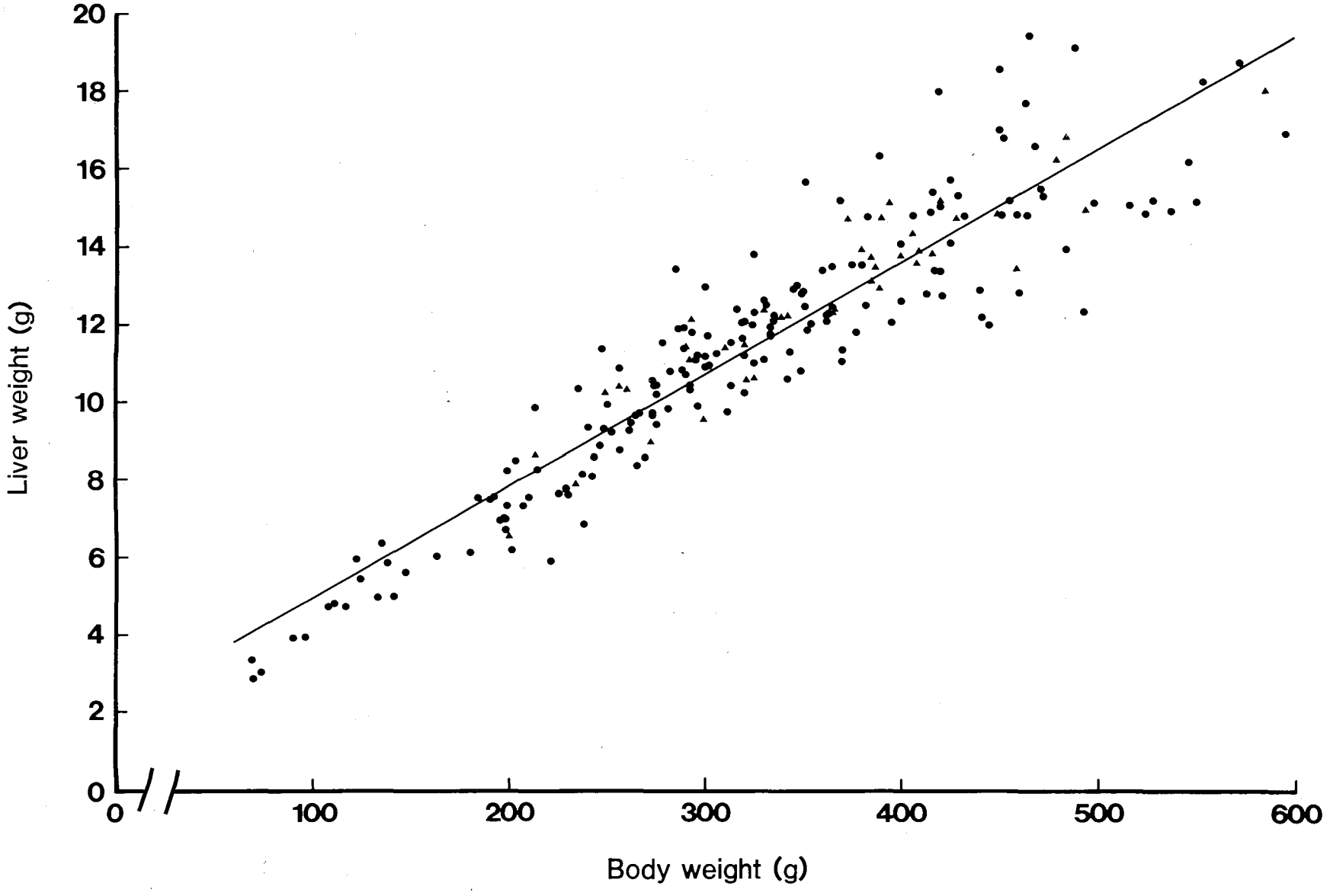
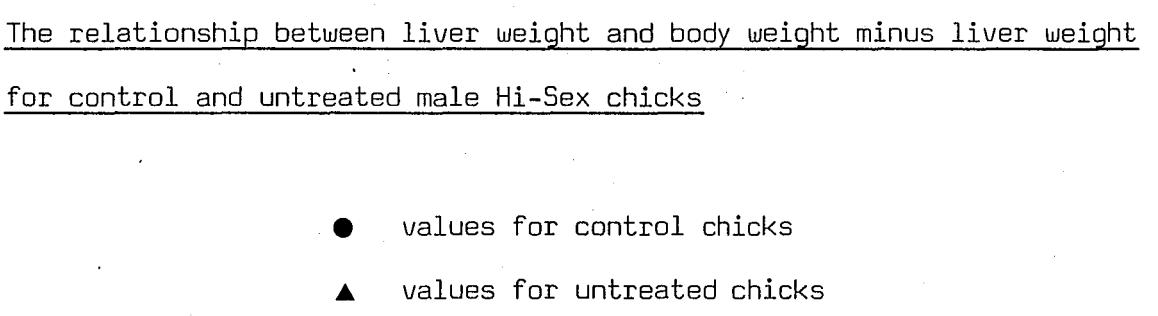

Liver weight (g)
20
18
16
14
12
10
8
6
4
2
0
0
100
200
300
400
500
600
Body weight (g)

FIGURE 2



Control chicks had received a single intramuscular injection of propane-1,2-diol (0·1 ml/100 g body wt.) at varying times prior to sacrifice.

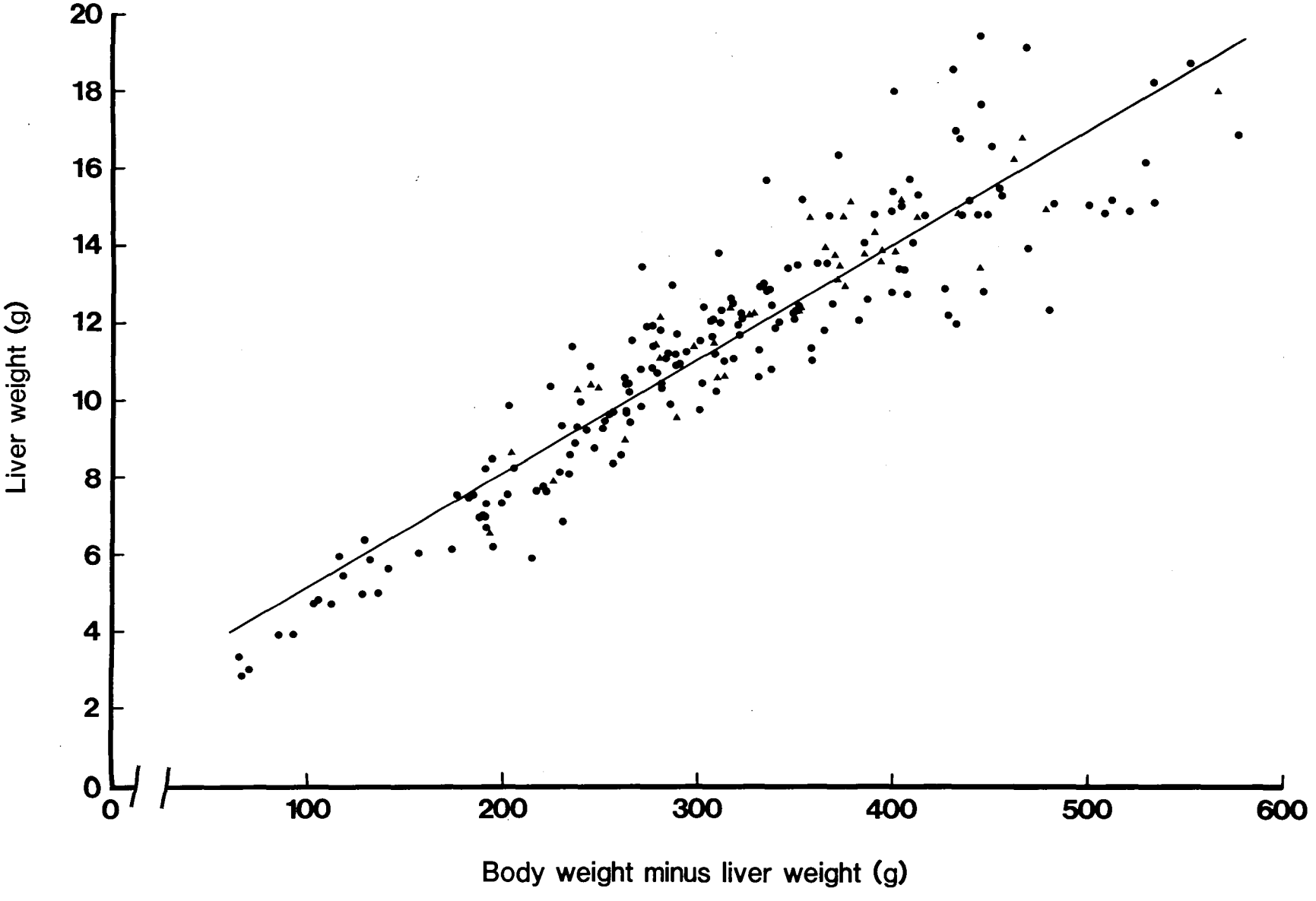
Regression equation:-

$$y = 0{\cdot}030x + 2{\cdot}194$$

where y = liver weight (g) and x = body weight minus liver weight (g)

Chicks were aged 2-6 weeks.

Separate regression analyses for control and untreated birds showed that there was no significant difference between these groups.


Liver weight (g)
20
18
16
14
12
10
8
6
4
2
0
0
100
200
300
400
500
600
Body weight minus liver weight (g)

chicks. In this case, the regression equation for the data is:-

$$y = 0{\cdot}030x + 2{\cdot}194$$

where y = liver weight (g) and x = body weight minus liver weight (g). The high degree of positive correlation between liver weight and body weight minus liver weight is corroborated by the statistically significant correlation coefficient of 0·923 ($P < 0{\cdot}001$).

A common way of expressing organ weights is as percentages of body weight rather than as absolute values. This method is particularly useful when comparing organ weights of animals with different body weights, since the differing body weights are taken into consideration. However, this procedure of comparison is not as simple as it might at first appear, since organ weights are often not found to increase in direct proportion with total body weight. In the present study, this phenomenon has been observed with liver weight during the first few weeks of life of the male chick. The data presented in Fig. 3 illustrate the relationship between liver weight (as % of body wt.) and body weight for male Hi-Sex chicks aged 2 - 6 weeks. It is clear from this figure that, as the body weight increases, the liver weight comes to represent a lesser proportion of the total body weight. The regression equation for these data is:-

$$y = 4{\cdot}324 - 0{\cdot}002x$$

where y = liver weight (as % of body wt.) and x = total body weight (g). The correlation coefficient of -0·555 is statistically significant ($P < 0{\cdot}001$), indicating a high degree of negative correlation between liver weight (as % of body wt.) and total body weight. A similar relationship has been described by Raheja *et al.* [359] in male White Leghorn chicks over the same age range.

Several investigators have observed increases in liver weight after oestrogen treatment of chicks, and have expressed the liver weights as a percentage of body weight or as a proportion of body weight, without

FIGURE 3

The relationship between liver weight (as % of body wt.) and body weight of control and untreated male Hi-Sex chicks

● values for control chicks

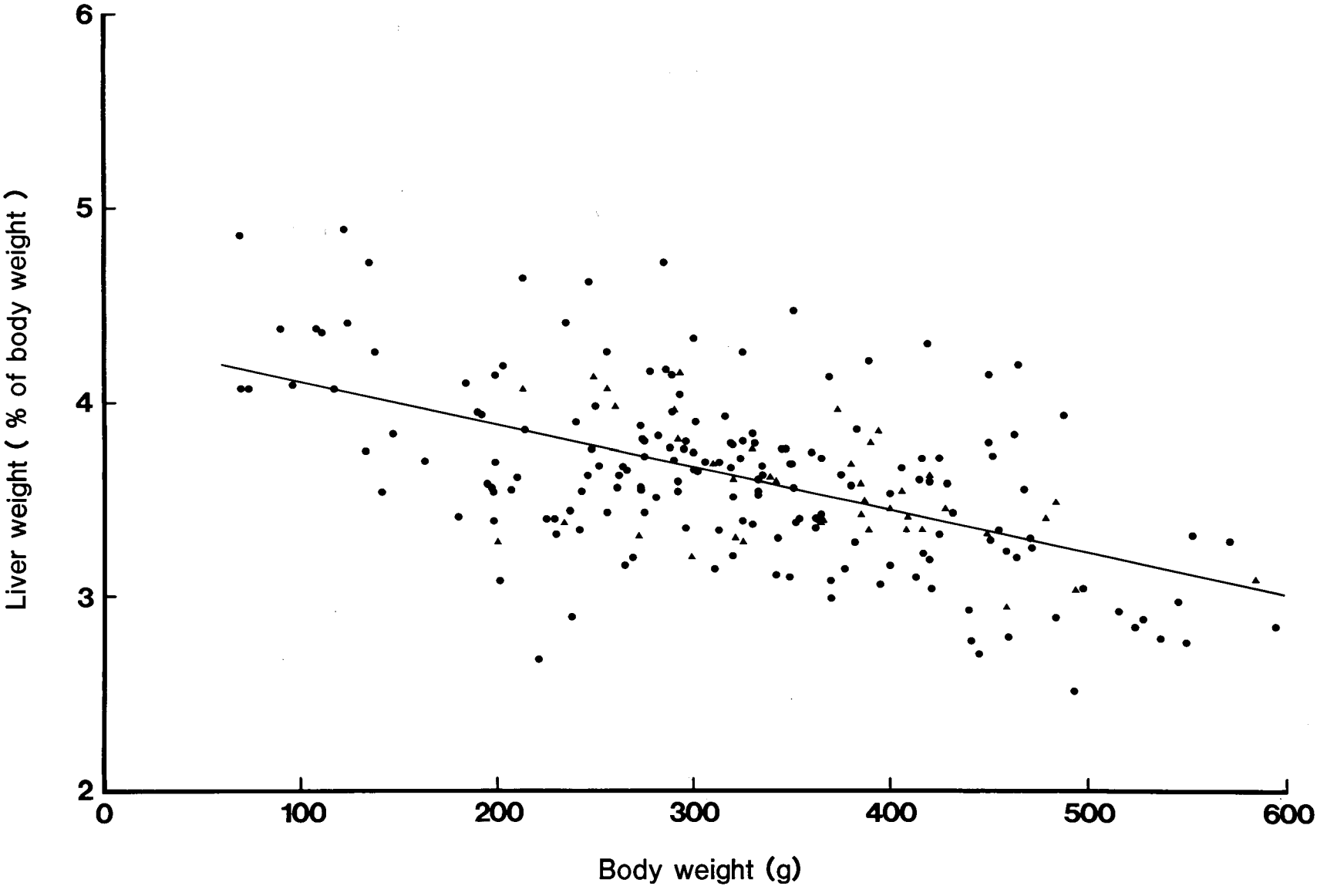
▲ values for untreated chicks

Control chicks had received a single intramuscular injection of propane-1,2-diol (0·1 ml/100 g body wt.) at varying times prior to sacrifice.

Regression equation:-

$$y = 4{\cdot}324 - 0{\cdot}002x$$

where y = liver weight (as % of body wt.) and x = total body weight (g)


Liver weight (% of body weight)
6
5
4
3
2
0
100
200
300
400
500
600
Body weight (g)

taking into account the normal variation in these parameters during the growth of chicks [166, 256]. In the present study, birds were injected with 1 mg 17β-oestradiol/100 g body weight, and were sacrificed at varying times thereafter. Using the regression equation derived from the data presented in Fig. 2, the liver weight of an oestrogenized chick could be compared with the expected liver weight for a control or untreated bird of the same body minus liver weight. The results of such analyses are presented in Fig. 4, in which the percentage change in liver weight is plotted against the time after 17β-oestradiol injection. These data clearly show increased liver weights with increasing time after hormone injection. A 50% increase in liver weight became evident after about 20 hours post-injection, and after about 40 hours some liver weights were found to be double those of equivalent control birds. Peak values were obtained at about 50 hours after injection, when liver weights were up to 2·4-fold greater than the weights expected if the chicks had not been treated with oestrogen. At times later than 50 hours after injection the response appeared to be depressed. The livers of oestrogen-treated birds became noticeably larger, paler, fatty in appearance, and softer in texture with increasing time after 17β-oestradiol injection up to 50 hours.

The data presented in Table 2 were obtained from an experiment in which each male chick received a single intramuscular injection of 17β-oestradiol (0·25 - 1·25 mg/100 g body wt.) in propane-1,2-diol, and birds were sacrificed 48 hours after injection. Untreated birds, and control birds injected with an equivalent volume of propane-1,2-diol only, were also included. It can be clearly seen that there is a dose-related increase in liver weight, as a percentage of body weight, over the dose range used. This trend is also evident when the varying body weights of the birds are taken into account and the results are expressed

FIGURE 4

The percentage change in liver weight of male Hi-Sex chicks at varying times after a single injection of 17 β-oestradiol (1 mg/100 g body wt.)

Each chick received a single intramuscular injection of 1 mg 17 β-oestradiol (in propane-1,2-diol)/100 g body weight. At the indicated times after injection birds were sacrificed, and livers were removed and weighed.

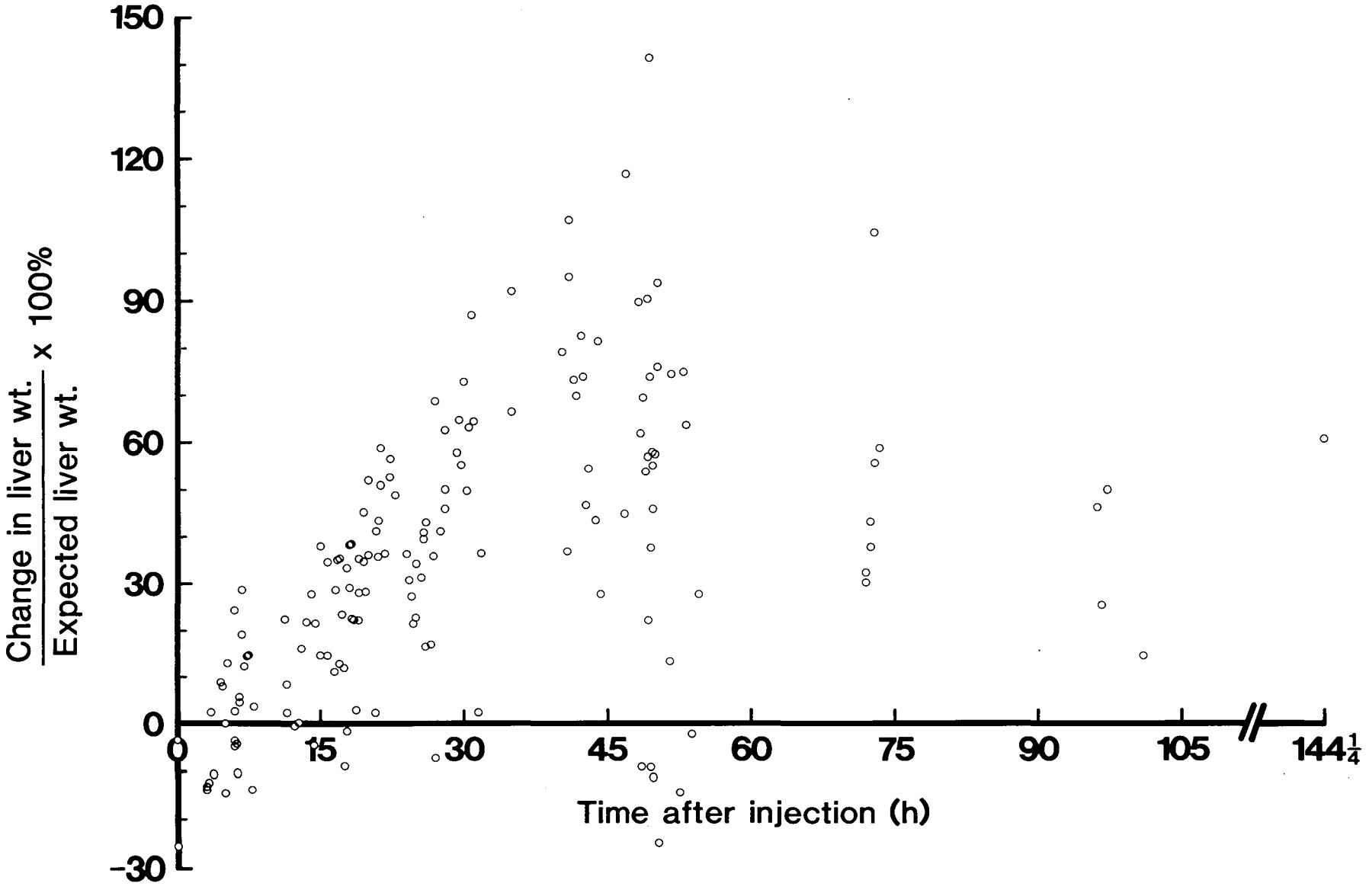

150
120
90
60
30
0
-30
0
15
30
45
60
75
90
105
144¼
Change in liver wt. / Expected liver wt. x 100%
Time after injection (h)

TABLE 2

The effect of varying doses of 17 β-oestradiol on the liver weights of male chicks sacrificed 2 days after injection

	17 β-oestradiol administered (mg/100 g body wt.)						
	0 Control	0 * Untreated	0·25	0·50	0·75	1·00	1·25
Body weight (g)	412 ± 15	395 ± 5	299 ± 7	348 ± 5	394 ± 17	396 ± 12	390 ± 22
Liver weight (as % of body wt.)	3·51 ± 0·22	3·46 ± 0·05	3·97 ± 0·07	4·42 ± 0·12	5·00 ± 0·14	5·43 ± 0·12	6·09 ± 0·21
% increase in liver weight	——	——	11·02 ± 1·85	27·65 ± 3·79	48·15 ± 4·99	62·01 ± 4·11	82·10 ± 8·35
Age of chicks (days)	37	36	31	32	33	34	35

Values are the means (± S.E.M.) of 6 observations.

*These values are the means (± S.E.M.) of 5 observations.

as the percentage increase in liver weight caused by oestrogenization. With a dose of 0·75 mg 17 β-oestradiol/100 g body weight, liver weight increased by about 48% above that expected for untreated or control birds of the same size, whilst a dose of 1·25 mg hormone/100 g body weight resulted in an approximately 82% increase in liver weight.

Subsequent experiments, in which male chicks were treated with a single intramuscular injection of 0·75 mg 17 β-oestradiol/100 g body weight and sacrificed at varying times thereafter, illustrated a time-related increase in liver weight as a percentage of body weight (Tables 3 & 4). In the experiment presented in Table 3, birds were injected with 17 β-oestradiol in propane-1,2-diol and were killed between 3 and 40 hours later. Untreated chicks were also included. An exception to the trend of a time-related increase in liver weight (as % of body wt.) is the mean value obtained at 14 hours after hormone injection which is lower than the value obtained at 6 hours. It should be noted that the chicks in this 14-hour group had the greatest body weights and, therefore, from the relationship between body weight and liver weight (as % of body wt.) presented in Fig. 3, it would be expected that the liver weights of untreated and control chicks of this size would represent a lesser proportion of the total body weight than would be the case for smaller birds. Therefore, the response of the livers of these 14-hour birds may be camouflaged somewhat by presenting the liver weight data as a percentage of body weight. Consequently, the liver weight data were re-analyzed using the regression equation derived from the data illustrated in Fig. 2, and were presented as the percentage increase in liver weight after oestrogenization. A similar time-related trend was obtained after this treatment of the liver weight data, with the mean value at 14 hours after injection being slightly lower than the value at 6 hours. Comparison of the liver weight results of Table 3 with similar

TABLE 3

Liver weights and body weights of male chicks sacrificed at varying times after a single injection of 0·75 mg 17 β-oestradiol in propane-1,2-diol/100 g body weight

	Time after 17 β-oestradiol injection (h)						
	0 Untreated	3	6	14	19	27	40
Body weight (g)	380 ± 36	290 ± 10	316 ± 14	405 ± 20	340 ± 14	385 ± 25	295 ± 10
Liver weight (as % of body wt.)	4·21 ± 0·47	4·23 ± 0·19	4·66 ± 0·17	4·43 ± 0·14	5·10 ± 0·27	5·23 ± 0·11	5·59 ± 0·30
% increase in liver weight	—	17·58 ± 5·24	32·13 ± 4·41	31·16 ± 4·63	47·45 ± 7·84	54·65 ± 3·95	57·93 ± 9·52
Age of chicks (days)	34	28	30	35	32	33	29

Values are the means (± S.E.M.) of 5 observations.

TABLE 4

Liver weights and body weights of male chicks sacrificed at varying times after a single injection of 0·75 mg 17 β-oestradiol in propane-1,2-diol/100 g body weight or of propane-1,2-diol only

	Time after injection (h)								
	Untreated*	3		6		14		26	
		E	C	E	C	E	C	E	C
Body weight (g)	396 ± 9	368 ± 31	393 ± 16	405 ± 22	400 ± 22	320 ± 15	323 ± 20	344 ± 18	358 ± 7
Liver weight (as % of body wt.)	3·54 ± 0·06	4·00 ± 0·22	3·86 ± 0·13	4·01 ± 0·10	3·57 ± 0·11	4·54 ± 0·18	3·61 ± 0·06	5·03 ± 0·26	3·59 ± 0·17
% increase in liver weight	—	15·57 ± 5·67	—	18·26 ± 3·05	—	29·30 ± 5·49	—	45·89 ± 9·12	—
Age of chicks (days)	35	35	35	34	34	30	30	31	31

E = values for chicks injected with 0·75 mg 17 β-oestradiol in propane-1,2-diol/100 g body weight.

C = values for chicks injected with propane-1,2-diol only.

Values are the means (± S.E.M.) of 5 observations.

*These values are the means (± S.E.M.) of 4 observations.

results in Table 4 indicate that the abnormal value in Table 3 may, in fact, be the 6-hour value and not the 14-hour value. It should be noted at this stage that birds differed a great deal in the response of their liver weights to oestrogen treatment, as shown in Fig. 4. It can be seen that after oestrogen treatment some birds responded with a dramatic increase in liver weight, whilst some responded to a lesser extent and others appeared not to respond at all. The data presented in Table 3 show that at 3 hours after 17 β-oestradiol injection the mean liver weight had increased by about 18% above that expected for untreated or control birds of the same size, whilst by 40 hours the increase was approximately 58%.

The data presented in Table 4 resulted from an experiment in which male chicks were given a single intramuscular injection of 0·75 mg 17 β-oestradiol in propane-1,2-diol/100 g body weight, or of propane-1,2-diol only, and were sacrificed between 3 and 26 hours later. Untreated birds were also included. A time-related increase in liver weight (as % of body wt.) after oestrogenization was evident over this time range, although the mean value at 6 hours was virtually identical to the mean value at 3 hours after injection. At 14 and 26 hours after oestrogen treatment the mean liver weights (as % of body wt.) were progressively larger. The birds in the 6-hour oestrogen-treated group were heavier than the birds in the other oestrogen-treated groups, and therefore, as for the 14-hour group in Table 3, the response of the livers of these 6-hour birds may be concealed to a certain extent by presenting the liver weight data as a percentage of body weight. This is confirmed by expressing the liver weight data as the percentage increase in liver weight after oestrogenization, since the mean value for the 6-hour oestrogenized group is then noticeably greater than the value for the 3-hour group. In this experiment, the mean liver weight had increased by about 18% at

6 hours after oestrogen injection and by almost 46% at 26 hours. A similar time-related increase in liver weight (as % of body wt.) was not observed for control birds, confirming that the phenomenon of increasing liver weight was the result of oestrogen treatment and was not caused by handling and/or injection of propane-1,2-diol.

2. DNA content of the liver after oestrogen treatment

The data presented in Fig. 5 illustrate the relationship between the liver weight and DNA content of the liver for control and untreated male Hi-Sex chicks. The regression equation for these data is:-

$$y = 3{\cdot}837x - 7{\cdot}604$$

where y = DNA content of liver (mg) and x = liver weight (g). The correlation coefficient of 0·875 is statistically significant ($P < 0{\cdot}001$), indicating a high degree of positive correlation between the DNA content of the liver and the liver weight.

The increase in DNA content with increasing liver size represents DNA replication and cell division and a resulting increase in the number of liver cells, but may also reflect an increase in the number of polyploid cells.

The increase in the liver weights of chicks after oestrogenization involves some cell division, but a substantial part of the increase in liver size and weight is considered to be due to the increase in volume of pre-existing cells as a result of water uptake and accumulation of lipid [242]. The data presented in Fig. 6 show the DNA contents of the livers (mg DNA/g liver) of chicks sacrificed at varying times after a single intramuscular injection of 1 mg 17 β-oestradiol in propane-1,2-

FIGURE 5

The relationship between liver weight and DNA content of the liver for control and untreated male Hi-Sex chicks

● values for control chicks

▲ values for untreated chicks

Control chicks had received a single intramuscular injection of propane-1,2-diol (0·1 ml/100 g body wt.) at varying times prior to sacrifice.

Regression equation:-

$$y = 3{\cdot}837x - 7{\cdot}604$$

where y = DNA content of liver (mg) and x = liver weight (g)

Chicks were aged 3 – 6 weeks.

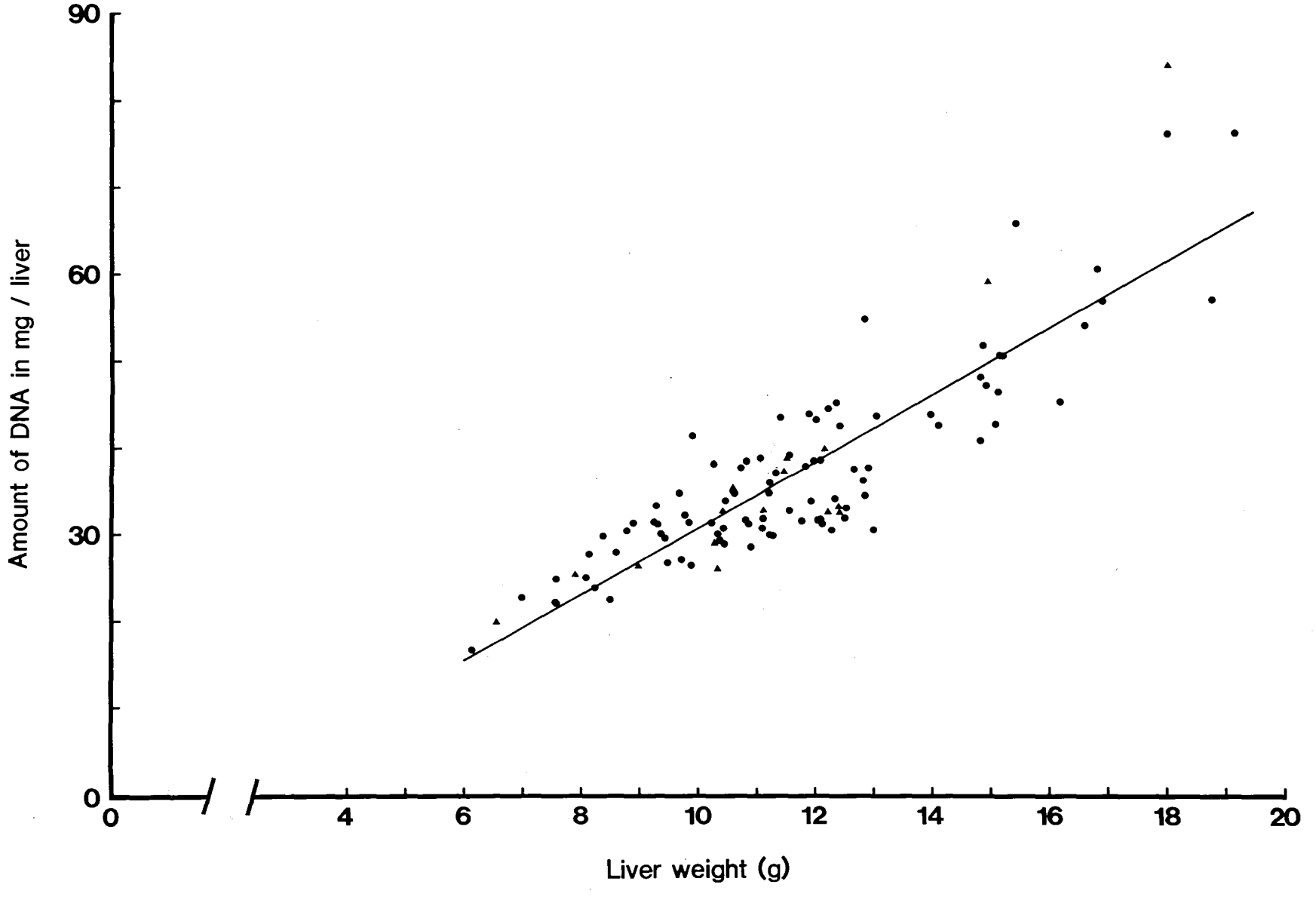

90
60
30
0
Amount of DNA in mg / liver
0
4
6
8
10
12
14
16
18
20
Liver weight (g)

FIGURE 6

The relationship between the liver DNA content (mg/g liver) of male Hi-Sex chicks, injected with 17 β-oestradiol in propane-1,2-diol or with propane-1,2-diol only, and time after injection

Oestrogen-treated chicks received a single intramuscular injection of 1 mg 17 β-oestradiol in propane-1,2-diol/100 g body weight at varying times prior to sacrifice. Control chicks received an equivalent volume of propane-1,2-diol only and were sacrificed at the times indicated.

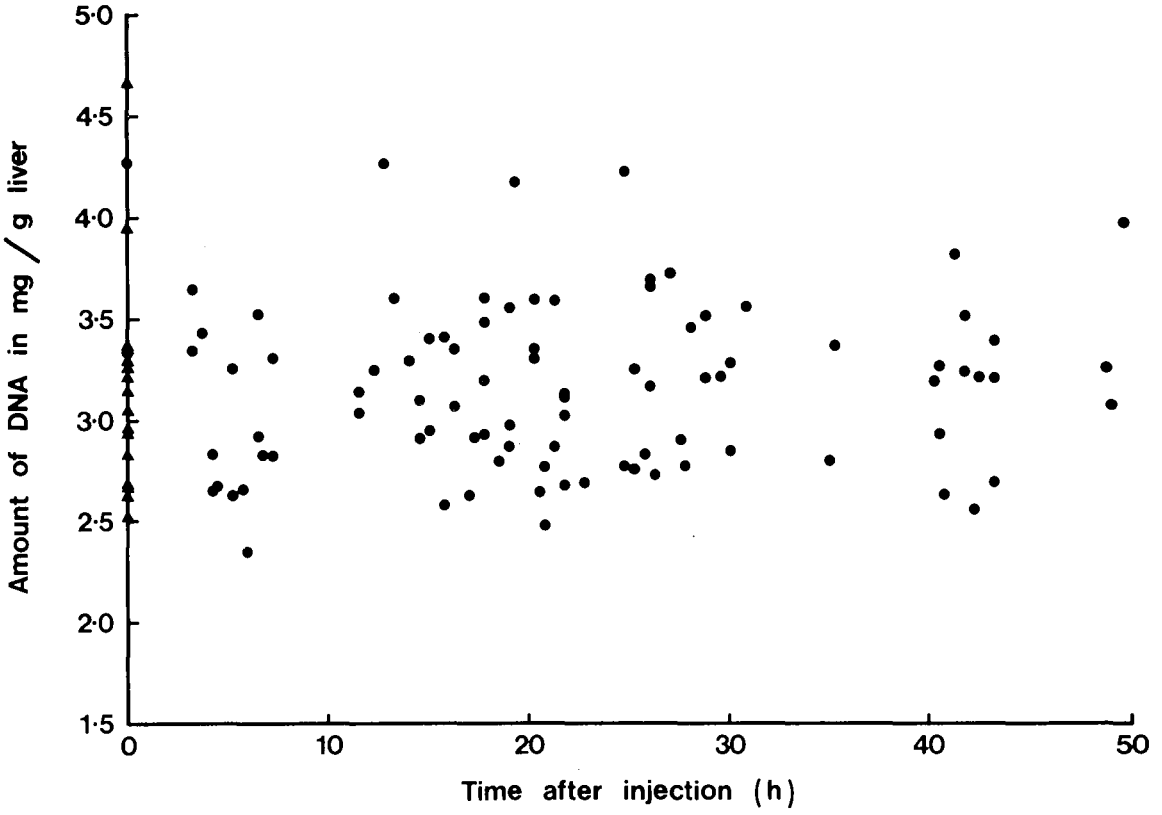
(a) ▲ values for untreated chicks

● values for control chicks

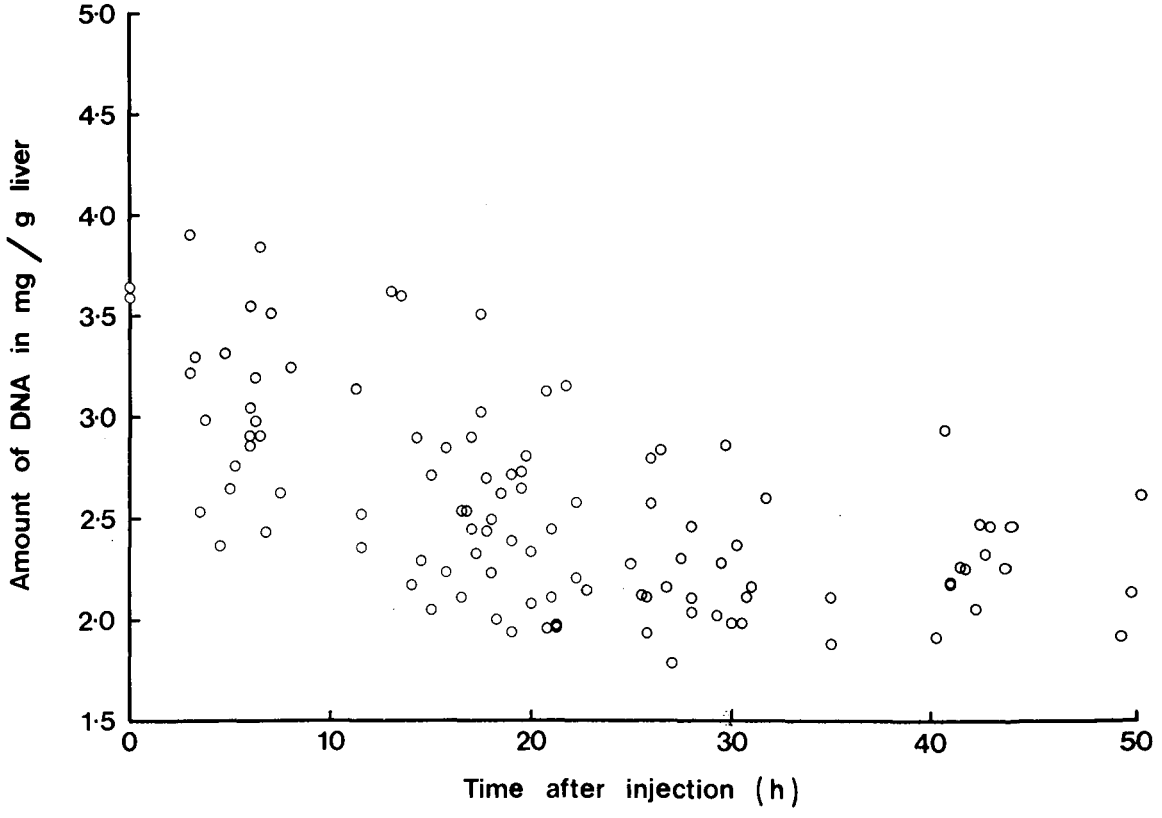
(b) ○ values for oestrogen-treated chicks

DNA was precipitated, washed and extracted from liver samples as described in the Methods section, and was assayed by a minor modification of the method of Burton (353).

(a)


5·0
4·5
4·0
3·5
3·0
2·5
2·0
1·5
Amount of DNA in mg / g liver
0
10
20
30
40
50
Time after injection (h)

(b)


5·0
4·5
4·0
3·5
3·0
2·5
2·0
1·5
Amount of DNA in mg / g liver
0
10
20
30
40
50
Time after injection (h)

diol/100 g body weight, or of an equivalent volume of propane-1,2-diol only. A time-related decrease in the DNA content of a unit weight of wet liver is evident for chicks that had received 17β-oestradiol at varying times up to 50 hours before death. A time-related decrease in liver DNA content (mg/g liver) after injection was not evident for control birds, indicating that the decrease in DNA content of a unit weight of wet liver was the result of oestrogen treatment and not of handling and/or injection of propane-1,2-diol. The liver DNA content for control and untreated birds ranged from 2·35 to 4·66 mg/g liver, with a mean value of 3·16 ± 0·04 (S.E.M.).

The data presented in Fig. 7 illustrate the relationship between the percentage change in the liver weights of chicks after oestrogenization and the DNA content of the liver (mg/g liver). It is evident that as the liver weight increases after oestrogen treatment the DNA content of a unit weight of the wet liver decreases, confirming that cell expansion contributes substantially to the increase in liver weight. The regression equation for these data is:-

$$y = 124{\cdot}909 - 34{\cdot}951x$$

where y = the percentage change in liver weight and x = the liver DNA content (mg/g liver).

The correlation coefficient of -0·630 is statistically significant ($P < 0{\cdot}001$), indicating a high degree of negative correlation between the percentage increase in liver weight and the DNA content of a unit weight of the liver. The lowest liver DNA content recorded was 1·79 mg/g liver, which was the result for a chick injected with 1 mg 17β-oestradiol/100 g body weight 27 hours before sacrifice. The liver weight of this chick was approximately 69% greater than that expected for an untreated or control chick of the same size, and represented 5·68% of the total body weight.

FIGURE 7

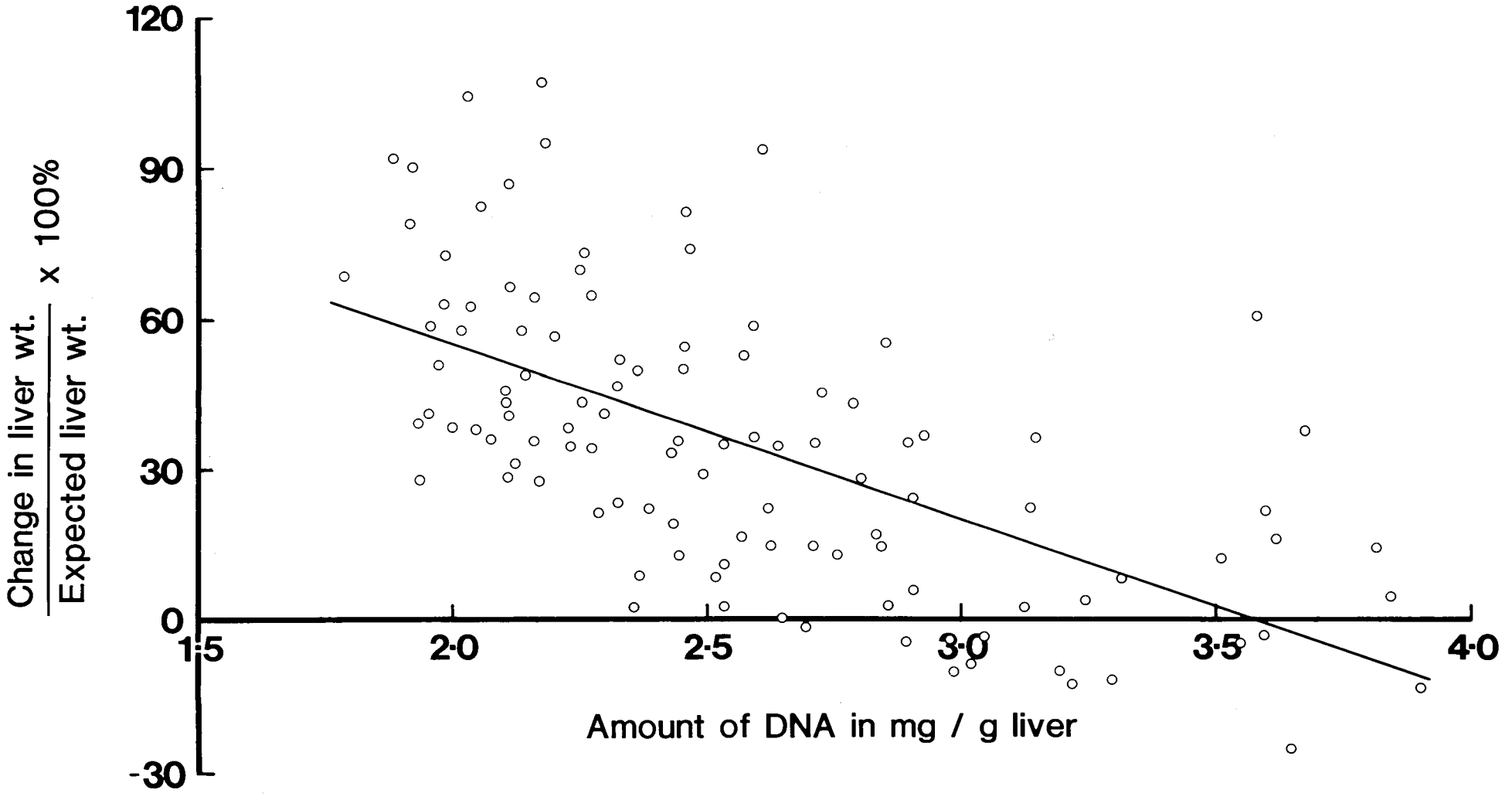
The relationship between the percentage change in liver weight of male Hi-Sex chicks caused by oestrogen treatment and the DNA content of the liver (mg/g liver)

Chicks received a single intramuscular injection of 1 mg 17β-oestradiol in propane-1,2-diol/100 g body weight at varying times before sacrifice. After killing, livers were removed, weighed and samples were taken for the assay of DNA. DNA was precipitated, washed and extracted from liver samples as described in the Methods section, and was assayed by a minor modification of the method of Burton (353).

Regression equation:-

$$y = 124{\cdot}909 - 34{\cdot}951x$$

where y = percentage change in liver weight after oestrogen treatment and x = liver DNA content (mg/g liver)


120
90
60
30
0
-30
1·5
2·0
2·5
3·0
3·5
4·0
Change in liver wt. / Expected liver wt. x 100%
Amount of DNA in mg / g liver

3. Plasma triacylglycerol levels after oestrogen treatment

Besides the changes in liver size and weight resulting from oestrogen treatment of male chicks which are readily apparent to the investigator, another noticeable change occurring after oestrogenization is the development of a cream-coloured clouding of the plasma. This turbidity is caused by accumulating lipid in the plasma, the bulk of which is triacylglycerol existing predominantly in the form of VLDL, which originate from the liver and are the result of enhanced hepatic VLDL synthesis (113 - 115, 118, 236, 242, 252). The data presented in Fig. 8 show the plasma triacylglycerol concentrations of male chicks at varying times after a single intramuscular injection of 1 mg 17 β-oestradiol in propane-1,2-diol/100 g body weight or of an equivalent volume of propane-1,2-diol only. The plasma triacylglycerol concentrations for control and untreated chicks ranged from 15 to 334 mg/100 ml plasma, with a mean value of 94·3 ± 4·7 (S.E.M.). These values are comparable with those presented for control and untreated chicks by other research workers (46, 113, 117, 174, 254, 360, 361). In the present study, the plasma concentrations of triacylglycerol for oestrogen-treated birds began to diverge noticeably from control values after about 10 hours post-injection, and increased progressively up to 50 hours after 17 β-oestradiol injection (Fig. 8). No such increase was evident for control birds. The values for oestrogenized chicks first became significantly greater than control values at 5 - 7½ hours post-injection ($P < 0{\cdot}05$). The highest plasma triacylglycerol concentration was obtained for a chick injected with 1 mg 17 β-oestradiol/100 g body weight at approximately 49 hours before sacrifice, in which the value reached 3070 mg/100 ml plasma. This represents an approximately 33-fold increase in the triacylglycerol concentration of the plasma as compared with the mean control value.

FIGURE 8

Concentration of triacylglycerol in the plasma (mg triacylglycerol/ 100 ml plasma) of male Hi-Sex chicks at varying times after injection of 17 β-oestradiol in propane-1,2-diol or of propane-1,2-diol only

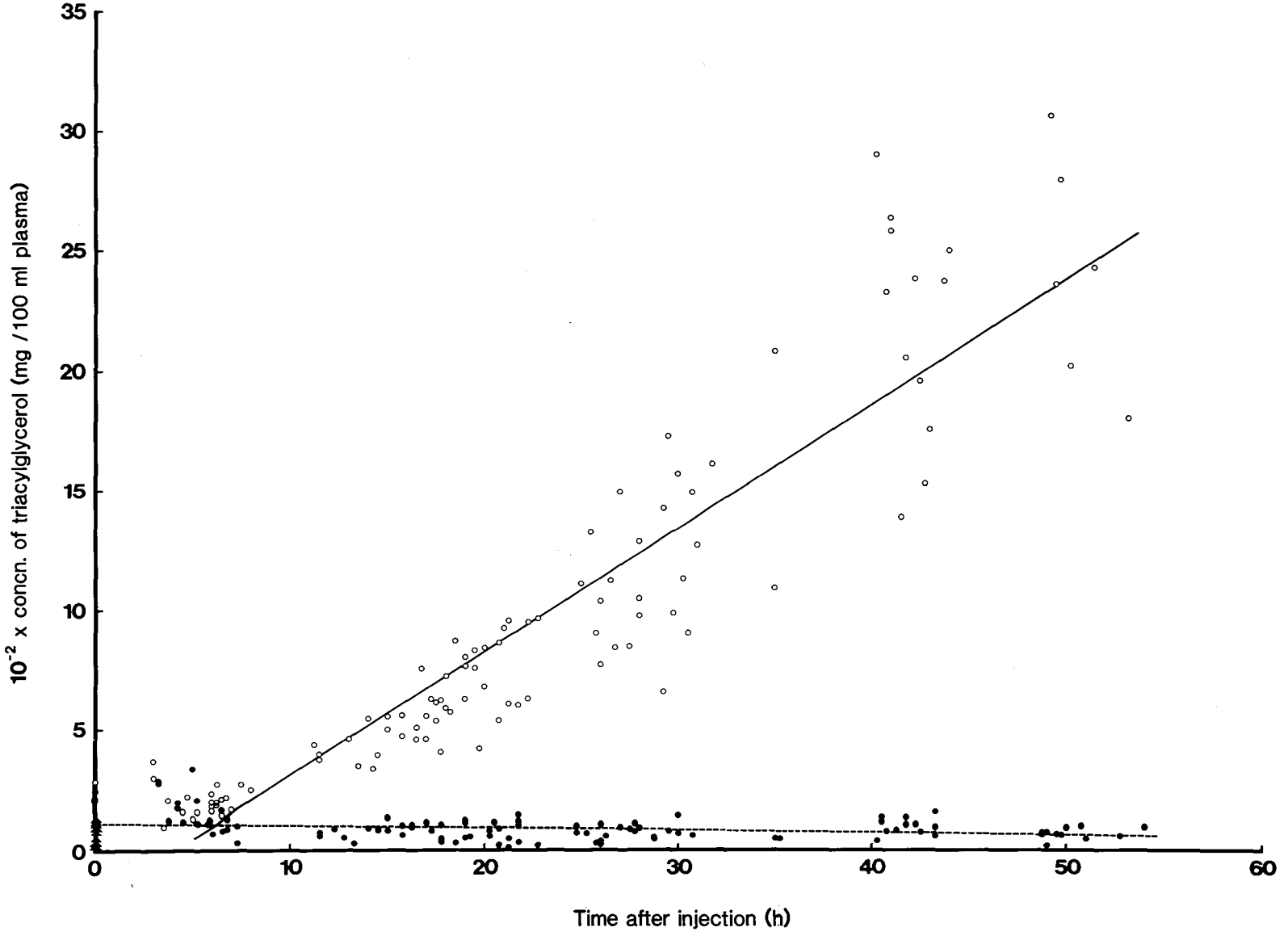
Oestrogen-treated chicks received a single intramuscular injection of 1 mg 17 β-oestradiol in propane-1,2-diol/100 g body weight at varying times prior to sacrifice. Control chicks received an equivalent volume of propane-1,2-diol only and were sacrificed at the times indicated.

▲ values for untreated chicks

● values for control chicks

○ values for oestrogen-treated chicks

Blood was collected and plasma was prepared as described in the Methods section. Total lipids were extracted from the plasma samples using the method of Bligh & Dyer (356). Triacylglycerol levels in the lipid extracts were determined as described in the text, using the Sigma Diagnostic Kit procedure detailed in the Sigma Technical Bulletin No. 405 (355).


35
30
25
20
15
10
5
0
10^{-2} x concn. of triacylglycerol (mg /100 ml plasma)
0
10
20
30
40
50
60
Time after injection (h)

4. Plasma protein-bound phosphate levels after oestrogen treatment

Another plasma parameter that changes after oestrogen treatment of chicks is the concentration of protein-bound phosphate. The results presented in Fig. 9 clearly show that at about 17½ hours after 17 β-oestradiol injection (1 mg hormone/100 g body wt.) the plasma concentration of protein-bound phosphate begins to diverge from control values, and increases progressively up to about 42½ hours. Values remained high up to 55 hours post-injection, which was the longest time investigated. Values for oestrogenized chicks first became significantly greater than control values at 14 - 18 hours post-injection ($P < 0{\cdot}02$). The only phosphoprotein present in any significant amount in the plasma of oviparous vertebrates is vitellogenin [81, 250, 330], and therefore, the concentration of phosphate covalently bound to plasma proteins can be used as an estimate of the concentration of vitellogenin in plasma. The plasma protein-bound phosphate concentrations for control and untreated chicks ranged from 0·17 to 0·43 μmoles/ml plasma, with a mean value of 0·26 ± 0·01 (S.E.M.). The highest plasma concentration of protein-bound phosphate was obtained for a chick injected with 1 mg 17 β-oestradiol/100 g body weight at approximately 42 hours before sacrifice, in which the value reached 2·10 μmoles/ml plasma. This represents an approximately 8-fold increase in the concentration of protein-bound phosphate in the plasma as compared with the mean control value.

FIGURE 9

Concentration of protein-bound phosphate in the plasma (μmol/ml plasma) of male Hi-Sex chicks at varying times after injection of 17 β-oestradiol in propane-1,2-diol or of propane-1,2-diol only

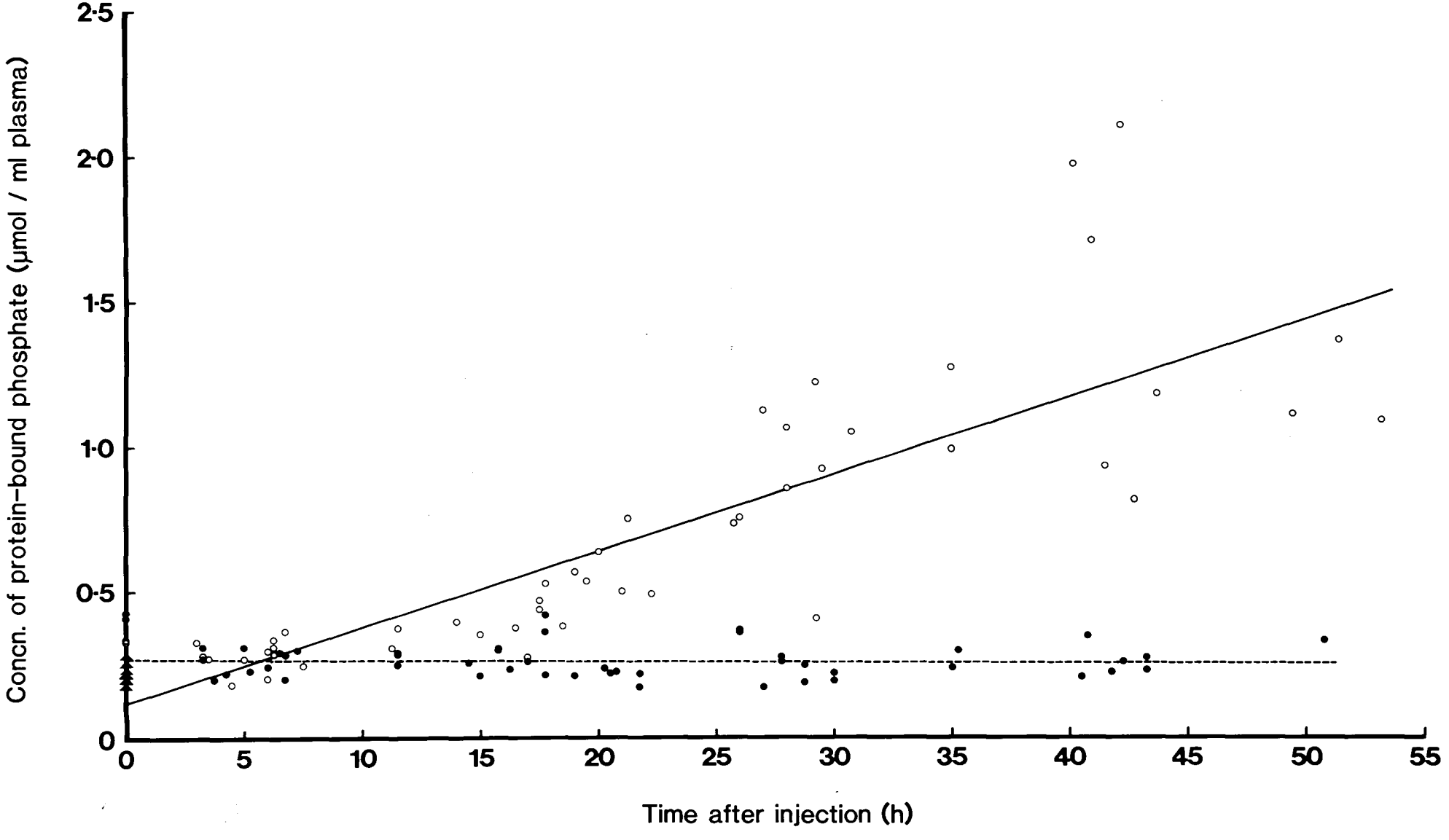
Oestrogen-treated chicks received a single intramuscular injection of 1 mg 17 β-oestradiol in propane-1,2-diol/100 g body weight at varying times prior to sacrifice. Control chicks received an equivalent volume of propane-1,2-diol only and were sacrificed at the times indicated.

▲ values for untreated chicks

● values for control chicks

○ values for oestrogen-treated chicks

Blood was collected and plasma was prepared as described in the Methods section. Plasma proteins were precipitated and lipid was removed from the protein precipitate as described in the text. The alkali-labile phosphate content of the lipid-free protein precipitate was determined by the method of Ames (357).


Concn. of protein-bound phosphate (µmol / ml plasma)
2·5
2·0
1·5
1·0
0·5
0
0
5
10
15
20
25
30
35
40
45
50
55
Time after injection (h)

DISCUSSION

1. Changes in liver weight during growth and after oestrogen treatment

The positive correlation between the liver weights and total body weights of untreated and control chicks, illustrated in Fig. 1, is in agreement with the observations of Matsuzawa (358), who has described the changes in weights of a number of organs during the first 20 weeks of life of White Leghorn chicks. The body weights of male birds of this breed were very similar to the body weights recorded in the present study for male Hi-Sex chicks of the same age. Matsuzawa observed an increase in liver weight in both sexes concurrently with increased body weight during the course of his study. The various organs differed somewhat in the time courses of their weight increases, with some increasing in weight rapidly over the first few weeks and then increasing more slowly latterly, whilst others increased in weight until a certain age after which they remained constant or decreased in weight. Differences in the weight increments of organs were also observed between the sexes. For example, heart weight increased in both sexes over the first 18 weeks, but after 6 weeks the heart weights of male birds were about 1·5-fold greater than those of female birds. Although the body weights of female birds were less than those of male birds after 4 weeks of age, the differences in body weight between the sexes were not as pronounced as the differences in heart weight. The brain weights of females were again less than those of males after 4 weeks of age, but in both sexes the brain weight increased up to 12 weeks and was constant thereafter. The weights of the pancreas and spleen increased rapidly during the first 10 weeks of life and increased more slowly after this. Two organs that increased in weight up to a certain age and then regressed were the bursa Fabricii and the thymus gland. The most rapid

increase in liver weight for chicks of both sexes was between 2 and 10 weeks of age.

The examples presented above are just a few representative observations noted by Matsuzawa, and illustrate the variability of the growth patterns of different organs with increasing age and body weight in the domestic fowl. These data show that organ weights may increase in proportion with the weight of the whole body at certain stages of development, but that this relationship may not be upheld at other times. This leads to difficulties as to the best way of expressing organ weights of growing animals, with different body weights, for comparison. Many workers have expressed organ weights as percentages or proportions of total body weight after exposing animals to various conditions which result in changes in organ weights (166, 250, 252, 256, 359, 360). Although the comparison of such values for animals of the same total body weight may be considered to be valid, the data presented in Fig. 3 show that liver weight, at least, declines as a percentage of total body weight as body weight increases in normal growing chicks. It is interesting at this point to note the similarity between these results and the results of Raheja et al. (359), who observed that in day old male White Leghorn chicks the liver weight represented a high proportion of body weight. This proportion increased still further during the first week of life, and then declined steeply to about 4 weeks of age and more gradually thereafter to a constant level at the 14th week.

It is well-known that oestrogen treatment of male and immature female birds results in increased liver weights some hours later (58, 124, 162, 166, 174, 242 - 248, 250), and similarly, endogenous oestrogens cause the livers of laying hens to be larger than those of non-laying hens and male birds (58, 125, 164). It would, therefore, be possible to treat a large chick with oestrogen and to observe an increase in

liver weight (as % of body wt.) compared with that of a control bird of the same body weight, but if compared with the liver weight (as % of body wt.) of a smaller control bird it might be assumed that no change had occurred if the relationship presented in Fig. 3 was not taken into account. It is, therefore, important to present data for body weights if expressing liver weights as a percentage or proportion of body weight. In the present study, chick liver weights after oestrogenization were expressed predominantly as percentage increases in liver weight as compared with the liver weights of control birds with the same body minus liver weight, since the body weights of the birds varied a great deal.

The data illustrated in Fig. 4 were deduced using the regression equation derived from the data presented in Fig. 2, in which the liver weights of control and untreated chicks were plotted against the values of body weight minus liver weight. This regression equation enables a more accurate calculation of the liver weights that would have been expected for oestrogen-treated chicks had oestrogen not been administered than that derived from the data presented in Fig. 1. This is because the livers of oestrogenized birds can contribute substantially more to the total body weight than do the livers of control and untreated birds, and from Fig. 1 no allowance can be made for this phenomenon. This procedure is based, however, on the assumption that the injection of 17β-oestradiol does not increase the weights of other organs in the male chick and lead to a general increase in body weight. This may not be the case, since some investigators have noted that oestrogenization increases the food intake of the domestic fowl [82, 174, 246, 362]. In addition, the large amounts of lipid produced by the liver of the oestrogenized male and immature female chicken accumulate in the blood, and some may be deposited in the adipose tissues which would consequently increase in weight. On the other hand, Coleman *et al.* [254] found no difference in the average body weight gain between control and oestro-

genized chicks after treating 6 to 11-day old chicks with 2 mg diethylstilbestrol in sesame oil, or with sesame oil only, each day for 5 days.

A time-related increase in the liver weights of chicks up to 50 hours after the injection of 1 mg 17 β-oestradiol/100 g body weight is shown in Fig. 4. A similar time-related increase in the liver weights of chicks was obtained up to 40 hours after the injection of 0·75 mg 17 β-oestradiol/100 g body weight (Tables 3 & 4). Other workers have obtained comparable trends for liver weight, comb weight and oviduct weight of the immature female chicken after treatment with gonadal hormones [166, 256, 363]. For example, Balnave & Pearce [166] injected 4-week old pullets with 2 mg oestradiol dipropionate on alternate days for 9 days, and presented organ weights as proportions of body weight. These workers did not obtain any difference in liver weight between control and oestrogenized birds during the first 12 hours after hormone treatment. However, after 24 hours and up to 4 days after the beginning of hormone treatment, liver weight was found to progressively increase. The response was reduced considerably by the 9th day of oestrogen treatment. Increases in liver weight were only observed for birds receiving oestrogen. Treatment of 4-week old pullets with 2 mg testosterone propionate on alternate days over a period of 9 days resulted in a time-related increase in comb weight, as a proportion of body weight, from the 2nd day until the end of the experiment. Both treatment with oestradiol as described above, and similar treatment of the immature pullets with 2 mg oestradiol dipropionate and 1 mg testosterone propionate together, produced a time-related increase in oviduct weight from 24 hours after hormone injection through to the 9th day of hormone treatment. On the 4th and 9th days of hormone treatment, the oviduct weights of the birds treated with oestradiol and testosterone were significantly greater than those of birds treated with oestradiol alone, illustrating

the enhancement of oestrogen-induced oviduct growth by testosterone. Pearce & Balnave [256] also noted time-related increases in oviduct weights and liver weights of 4-week old pullets treated with a variety of doses of oestradiol dipropionate (0·5 - 4 mg hormone/bird). Organ weights were determined 2 days after a single injection of oestrogen, and 8 days after the commencement of hormone treatment, during which time oestrogen was administered on alternate days. At all dose levels, both oviduct weight and liver weight were greater after 8 days than after 2 days of hormone treatment.

Time-related increases in liver weight have also been observed after oestrogen treatment of male birds of other species. For example, Gibbins & Robinson [250] injected 3 to 5-month old male Japanese quail with a single dose of 4·36 mg oestradiol/100 g body weight, and observed a near doubling of liver weight by the 4th day after oestrogen treatment, but the response decreased at longer time intervals. Similarly, Dashti et al. [252] treated 19-day old male turkeys with a single injection of diethylstilbestrol (4 mg/100 g body wt.) and observed a time-related increase in liver weight (as % of body wt.) up to 48 hours after injection, followed by a decreased response at 72 hours.

The data presented in Table 2 illustrate a dose-related increase in the liver weights of oestrogen-treated male chicks over the dose range 0·25 - 1·25 mg 17β-oestradiol/100 g body weight. Birds were injected with a single dose of hormone and were sacrificed 48 hours later. Similar dose-related trends in liver weight and oviduct weight were obtained by Pearce & Balnave [256], who injected 4-week old pullets with varying doses of oestradiol dipropionate (0·5 - 4 mg/bird) and sacrificed the birds 48 hours after a single injection or 8 days after the commencement of hormone treatment, during which time the birds received a hormone injection on alternate days. After 2 days of oestrogen

treatment, oviduct weight (as % of body wt.) increased up to a dose of 1 mg oestradiol dipropionate, and with the higher doses the increase in weight was less. After 8 days of oestrogen treatment, oviduct weight increased progressively with increasing dose level. Liver weight (as % of body wt.) after 2 days of oestrogen treatment increased up to a dose of 2 mg oestradiol dipropionate and the response was depressed with a dose of 4 mg. A similar response pattern was observed after 8 days of hormone treatment. As noted previously, Balnave [164] observed an 'over-reaction' of immature female chickens to exogenous oestrogen such that the liver weights were greater than those of mature laying hens. These observations suggest that large oestrogen doses and extended times of oestrogen treatment may lead to pharmacological effects rather than to a simulation of the situation existing in the laying hen. A decrease in liver weight after treatment with high oestradiol doses was not found in the present study, although a similar range of oestradiol dose levels was administered. The body weights of the birds used by Pearce & Balnave [256] for the 2-day hormone treatment experiment were approximately 250 g. Taking into account the different forms of oestradiol used by these workers and used in the present study, Pearce & Balnave [256] injected oestradiol up to a dose of about 1·13 mg/100 g body weight. With this dose the liver weight response was depressed compared with that observed with a dose of approximately 0·57 mg oestradiol/100 g body weight. Contrary to this, in the present study oestradiol was administered up to a dose level of 1·25 mg/100 g body weight without any sign of a reduced response at the higher dose levels. The difference between the results of these two studies may be related to the sexes of the birds used, since Pearce & Balnave [256] used immature females, whilst male chicks were used in the present study.

2. DNA content of the liver during growth and after oestrogen treatment

The relationship between the total DNA content of the liver and the liver weights of control and untreated male chicks, illustrated in Fig. 5, represents another instance of a relationship that could be easily over-simplified in the absence of adequate information. From the data in Fig. 5, it would appear that cell division is more important in bringing about an increase in weight as livers get larger, and that the accumulation of materials such as water, lipid and glycogen may contribute substantially to the increase in weight in smaller livers. In addition, the development of blood vessels and connective tissue at this stage may lead to these structures representing a large proportion of the smaller livers. These components may not represent such a large proportion of the larger livers, in which dividing parenchymal cells may contribute more substantially to the liver weight. On the other hand, or in addition to the possibility of the above differences between small and large livers, there may be an increased incidence of polyploidy as the liver enlarges. Somatic polyploidy has been shown to be a common developmental feature of endocrine target tissues, including vertebrate liver (364).

The mean value (± S.E.M.) for the DNA content of the livers of control and untreated chicks was derived from the data presented in Fig. 6(a) and was found to be 3·16 ± 0·04 mg/g liver. This mean value is similar to the mean value of 3·46 mg DNA/g liver quoted by Hawkins & Heald (236) for the 9 to 16-week old immature hen. The decrease in DNA content of a unit weight of male chick liver after oestrogen treatment (Fig. 6(b)) is in agreement with results obtained by Hawkins & Heald (236) for oestrogen-treated female chicks. These investigators injected 11-week old pullets with 2 mg oestradiol monobenzoate on alternate days for 7 days, and on average obtained an approximately 33%

reduction in the DNA content of a unit weight of liver. The lowest DNA value reported for an oestrogenized pullet in their study was 2·40 mg DNA/g liver, whereas the lowest in the present study was 1·79 mg/g liver. The 'DNA-lowering' effect of oestrogen is also evident in the liver of the laying hen aged 23 - 32 weeks, for which Hawkins & Heald [236] reported a mean value of 2·50 mg DNA/g liver. These results indicate that uptake of water and accumulation of lipid contribute substantially to the increase in liver size observed as the hen comes into lay and after oestrogen treatment of immature male and female birds.

3. Plasma triacylglycerol and protein-bound phosphate levels after oestrogen treatment

The development of turbidity in the plasma of oestrogen-treated chicks, resulting from the accumulation of lipid, first becomes evident about 12 hours after the injection of 1 mg 17 β-oestradiol/100 g body weight (Fig. 8). It is likely that this lipid is of hepatic origin and comprises predominantly triacylglycerol in the form of VLDL [113 - 115, 117, 118, 122, 236, 242]. The values for the plasma triacylglycerol concentration of control and untreated chicks obtained in the present study (15 - 334 mg/100 ml plasma) are similar to the levels observed by other research workers. For example, Kudzma et al. [113] presented a mean value of 27 mg triacylglycerol/100 ml plasma for chicks that had received an injection of sesame oil each day for 18 days from the age of 5 days. In another experiment, these workers injected chicks with sesame oil daily over 6 days, and observed plasma triacylglycerol levels ranging from 84 ± 6 to 236 ± 25 mg/100 ml plasma (means ± S.E.M.). Plasma triacylglycerol levels of 39 - 168 mg/100 ml were obtained by Kudzma et al. [117] for untreated 9-week old female chickens. Coleman et al. [254] injected 6 to 11-day old chicks with a daily dose of sesame

oil for 5 days, and recorded a serum triacylglycerol concentration of 151 ± 51 mg/100 ml (mean ± S.D.). Other workers have claimed sex differences in the levels of plasma triacylglycerol in chicks, and also differences with age. Pearson & Butler [174] injected male and female chicks aged 6 - 7 weeks with 5 doses of 0·5 ml 95% (v/v) ethanol given at 3-day intervals, and obtained mean plasma triacylglycerol levels (± S.E.M.) of 90 ± 7 mg/100 ml for female chicks, and 79 ± 10 mg/100 ml for male chicks that were fed ad libitum. Raheja [360] has reported plasma triacylglycerol levels for untreated chicks ranging from 85·7 ± 7·98 to 104·8 ± 4·97 mg/100 ml (means ± S.E.M.) for 5-week old chicks, and somewhat lower values of 70·4 ± 3·29 to 75·2 ± 4·93 mg/100 ml (means ± S.E.M.) for 14-week old chicks.

The treatment of chicks with 1 mg 17 β-oestradiol/100 g body weight, and sacrifice of the birds up to 55 hours after hormone administration, resulted in increased plasma triacylglycerol levels up to 33-fold greater than the mean level observed for control and untreated chicks, with the highest value reached being 3070 mg/100 ml plasma (Fig. 8). These increases are moderate compared with those obtained by other workers after chronic treatment of chickens with oestrogen. For example, Kudzma et al. [113] demonstrated a dose-related increase in plasma triacylglycerol levels after treating 5-day old chicks with 0·1, 1·0 or 5·0 mg diethylstilbestrol/day for 18 days. The mean level obtained with the 5 mg dose was 12,371 mg triacylglycerol/100 ml plasma, which represented an approximately 458-fold increase compared with the value for control chicks. However, other results obtained by these workers are lower than those obtained in the present study. For example, some chicks were injected with a daily dose of 0·5 mg diethylstilbestrol over a period of 6 days, and groups of birds were sacrificed 24 hours after each injection during this time. Plasma triacylglycerol

levels increased progressively with the duration of hormone treatment, and 24 hours after the 6th injection the mean triacylglycerol level (± S.E.M.) was 2288 ± 229 mg/100 ml plasma. This represented an approximately 27-fold increase above the mean value for control chicks.

Pearson & Butler [174] also showed a dose-related increase in plasma triacylglycerol concentration for female chicks aged 6 - 7 weeks which were treated with oestrogen and fed ad libitum. These birds received an injection of 0·25, 0·5 or 1 mg 17β-oestradiol dipropionate/100 g body weight at 3-day intervals until a total of 5 injections had been administered, and birds were sacrificed 2 days after the last injection. The highest dose of 17β-oestradiol dipropionate (1 mg/100 g body wt.) caused a substantial increase in plasma triacylglycerol concentration to a value of 4350 ± 489 mg/100 ml plasma (mean ± S.E.M.), which represents a 48-fold increase above the mean value for control chicks. The highest value obtained by these workers for the plasma triacylglycerol concentration of ad libitum-fed oestrogenized male chicks was 5870 ± 914 mg/100 ml plasma (mean ± S.E.M.), which was evident after the administration of 17β-oestradiol dipropionate (1 mg/100 g body wt.) at 3-day intervals on 4 occasions, and killing of the birds 2 days after the final dose. This represented an approximately 74-fold increase in the concentration of plasma triacylglycerol compared with the mean value for control male birds.

Coleman et al. [254] injected 6 to 11-day old chicks with a daily dose of 2 mg diethylstilbestrol for 5 days, and recorded a serum triacylglycerol level of 7313 ± 1769 mg/100 ml serum (mean ± S.E.M.). This represented an approximately 48-fold increase compared with the mean value obtained for control birds. Dashti et al. [365] observed a similar increase (43-fold) in the plasma triacylglycerol concentration of 19-day old male turkeys after treatment with diethylstilbestrol for

2 days. In a later publication, Dashti et al. [252] reported a 30-fold increase in the plasma triacylglycerol concentration of 19-day old male turkeys 24 hours after the injection of 4 mg diethylstilbestrol/100 g body weight, and this was elevated to a 55-fold increase by 48 hours. A 2·3-fold increase in plasma cholesterol was also observed by these workers at 48 hours after hormone treatment. It is interesting to note that the control plasma triacylglycerol concentrations of these turkeys were somewhat lower than values obtained by others for control domestic fowl chicks.

The treatment of 3-week old male chicks with 1 mg 17β-oestradiol by Chan et al. [206] resulted in increased plasma triacylglycerol levels, which reached a maximum of about 1400 mg/100 ml plasma at approximately 48 hours after hormone injection. Treatment of male chicks of the same age with 5 mg 17β-oestradiol resulted in a mean level of 2780 mg triacylglycerol/100 ml plasma after 48 hours [116]. These authors also observed an initial decrease in plasma triacylglycerol concentration over the first 2 or 3 hours after oestrogen administration [115, 116]. An interesting observation arising from the present study was the slight elevation in control plasma triacylglycerol concentrations up to about $6\frac{1}{2}$ hours after injection, which may have been caused by handling and/or injection of propane-1,2-diol.

The administration of oestrogens to male and immature female birds is considered to cause physiological changes similar to those existing in the laying hen. Kelley et al. [231] have recorded a mean plasma triacylglycerol level for laying turkeys of 1974 mg/100 ml plasma, which is somewhat lower than many of the values mentioned above for oestrogenized male and immature female chickens. Triacylglycerol levels, however, are undoubtedly higher than this in the hen just prior to the commencement of laying, when plasma lipids in general are at their highest levels [55, 57, 127, 236].

Increases in plasma triacylglycerol concentration are also observed in mammalian species treated with oestrogens [134, 366], and of particular interest are the elevations in plasma triacylglycerol levels observed in women receiving oestrogen treatment [133, 182 - 184, 207, 218, 219, 229]. Usually, contraceptive steroids cause only slight increases in fasting levels of plasma triacylglycerol, phospholipid and cholesterol, and the triacylglycerol concentration remains within the normal range, the upper limit of which is 150 mg triacylglycerol/100 ml plasma [184]. Gershberg et al. [207] reported an increase in serum triacylglycerol levels of about 21% after premenopausal women had received oral contraceptive treatment for 3 months. The effects of long-term oestrogen treatment of normal premenopausal women were recorded by Kekki & Nikkilä [218], who observed that after 4 years of treatment, the mean plasma triacylglycerol concentration was increased from about 75 to 125 mg triacylglycerol/100 ml plasma. Contrary to the situation in the majority of women, occasionally, in women with a pre-existing endogenous hypertriacylglycerolaemia, a massive increase in plasma triacylglycerol concentration occurs after oestrogen treatment. For example, Glueck et al. [367] treated a woman with familial type V hyperlipoproteinaemia with 3 mg diethylstilbestrol/day, and the level of plasma triacylglycerol rose to 5200 mg/100 ml plasma. A fortnight after finishing treatment the triacylglycerol level had declined to 852 mg/100 ml plasma. In another patient with mild endogenous hypertriacylglycerolaemia, Zorrilla et al. [182] observed a plasma triacylglycerol concentration of 221 mg/100 ml plasma, and after 6 months of combined oestrogen and progestagen treatment the level had reached 1948 mg triacylglycerol/100 ml plasma. Similar massive increases in plasma triacylglycerol levels have been observed in postmenopausal women on oestrogen supplementation, and Glueck et al. [219] have observed plasma triacylglycerol levels ranging from 222 to 3840 mg/100 ml plasma in such women.

The concentration of protein-bound phosphorus or phosphate in the plasma of birds has been used by many investigators as a measure of the concentration of vitellogenin, since this is the only phosphoprotein present in any significant amount in the plasma of oviparous vertebrates (81, 250, 330). The highly phosphorylated nature of the phosvitin moiety of the molecule provides a valuable marker for its detection and quantification. The data presented in Fig. 9 show a time-related increase in plasma phosphoprotein concentration from approximately 17½ hours to 42½ hours after treating male chicks with a single injection of 17 β-oestradiol (1 mg/100 g body wt.). From this figure it is evident that a lag period of about 17½ hours exists between the time of oestrogen injection and the appearance of detectable changes in phosphoprotein concentration, which is due to vitellogenin, in the blood. Similar lag periods have been observed by other workers after primary oestrogenization of male birds (81, 368). For example, Gruber (368) reported that cockerels of 2 kg body weight injected with 12·5 mg oestradiol exhibited a lag phase of 15 - 20 hours before the phosphoprotein content of the plasma was elevated. Jailkhani & Talwar (162) observed a lag phase of about 24 hours before the appearance of detectable amounts of vitellogenin in the circulation, after treating cockerels with 2 mg 17 β-oestradiol/100 g body weight. A similar lag period was observed by Mäenpää & Bernfield (335) after treating cockerels with a dose of 1 mg 17 β-oestradiol benzoate/100 g body weight. The length of the lag period obviously depends upon the sensitivity of the procedure used to determine the vitellogenin content of the blood. Gruber (243) injected oestrogen-treated cockerels with ^{32}P-phosphate at various times after the hormone injection, and measured the incorporation of radioactivity into phosvitin. This method is more sensitive than the chemical assay of protein-bound phosphate, and elevated plasma

phosphoprotein levels were observed after 5 - 7 hours using this technique, although vitellogenin synthesis starts 3 - 4 hours after hormone injection [81]. The lag phase is considered to be associated with transcription and translation of mRNA sequences coding for vitellogenin, followed by post-translational modification of the protein prior to secretion into the blood. These post-translational modifications occur in the endoplasmic reticulum and Golgi apparatus during the passage of the protein towards the cell surface. It is generally considered that phosphorylation of phosvitin occurs after translation [100, 101, 103, 104, 106], although some workers have suggested that phosphate is incorporated into the vitellogenin molecule by means of phosphoserine tRNAs [333, 334].

Comparison of Figs. 8 and 9 shows that the increase in plasma triacylglycerol concentration after oestrogen treatment of male chicks becomes evident several hours before an increase in protein-bound phosphate is detectable. This observation is in agreement with the reports of Bergink et al. [81] and Williams et al. [310], who observed that the rise in concentration of plasma VLDL in oestrogen-treated cockerels occurred earlier and lasted longer than the increase in vitellogenin concentration. The lag phase is independent of the oestrogen dose, but the duration of the rising phase, the maximum level of vitellogenin attained, and the descending phase are dose-dependent [163]. Beuving & Gruber [163] have shown that the maximum plasma phosphoprotein levels attained for oestrogenized 3 to 6-month old cockerels are essentially linearly related to the oestrogen dose, at least up to a dose of 20 mg 17β-oestradiol/animal.

The mean control level of protein-bound phosphate in the present study was 0·26 μmoles/ml plasma, which is equivalent to about 25 μg protein-bound phosphate/ml plasma or to about 8 μg protein-bound

phosphorus/ml plasma. It would seem likely that the phosphoprotein in control plasma is vitellogenin, since other workers have demonstrated the presence of vitellogenin at very low levels in the sera of non-oestrogenized cockerels, and have indicated that expression of the vitellogenin genes occurs at a basal rate prior to exogenous oestrogen treatment.[148, 150]. The maximum level of protein-bound phosphate obtained in the plasma of an oestrogen-treated chick in the present study was 2·10 µmoles/ml plasma, which is equivalent to about 200 µg protein-bound phosphate/ml plasma or to about 65 µg protein-bound phosphorus/ml plasma. This concentration of protein-bound phosphate was obtained for a chick that had received an injection of 17 β-oestradiol (1 mg/100 g body wt.) 42¼ hours before sacrifice. Vitellogenin has been shown to contain 3·4 ± 0·3% phosphorus by weight [64], and therefore, the concentration of vitellogenin in plasma from control chicks in the present study was approximately 216 - 258 µg/ml plasma, whilst the highest concentration attained for an oestrogen-treated chick was about 1757 - 2097 µg vitellogenin/ml plasma.

Gruber [368] observed a similar protein-bound phosphate level of about 210 µg protein-bound phosphate/ml plasma 100 hours after injecting 2 kg cockerels with 12·5 mg 17 β-oestradiol. Other workers have, however, observed much higher levels after treating cockerels with larger and repetitive doses of oestrogen. For example, Goldstein & Hasty [100] treated 2 - 2·3 kg cockerels with repetitive doses of 30 mg or 60 mg 17 β-oestradiol, and plasma levels of alkali-labile phosphorus were observed to reach approximately 360 µg/ml plasma. Similarly, Mäenpää & Bernfield [335] treated 1·8 - 2 kg cockerels with 1 mg 17 β-oestradiol benzoate/100 g body weight, and 5 days later the level of plasma protein-bound phosphorus was about 350 µg/ml plasma, representing a 150-fold increase over their control values. The

maximum amount of protein-bound phosphorus that has been detected in chicken plasma after oestrogenization is 360 - 420 µg/ml plasma [250], which has been found in the plasma of chickens injected with 2 - 3 mg oestradiol/100 g body weight [81, 100, 163, 369]. Even higher protein-bound phosphorus levels have been observed in the plasma of oestrogenized male and female quail that received 4·36 mg oestradiol/100 g body weight [250]. Control levels of protein-bound phosphorus were about 4 µg/ml plasma. The mean values for male and female quail 6 - 7 days after a single oestrogen dose were approximately 850 and 970 µg protein-bound phosphorus/ml plasma respectively, although values as high as 1500 µg/ml plasma were recorded. The vitellogenin of quail contains about half the amount of phosphorus/µg protein than does that of the chicken, showing that much higher levels of vitellogenin exist in the plasma of the quail than in the chicken after oestrogen treatment. The mean level of protein-bound phosphorus in laying female quail plasma was about 120 µg/ml plasma, which was raised to about 970 µg/ml plasma approximately 7 days after treatment with a single injection of 17 β-oestradiol (4·36 mg oestradiol/100 g body wt.). These abnormal levels of vitellogenin in the plasma were associated with cessation of laying and considerable stress, and are highly unphysiological.

The changes in the liver and the constituents of the plasma of male chicks after 17 β-oestradiol treatment described in this report were used to assay the response of the birds to the hormone. The results of subsequent experiments to elucidate the mechanisms of the increased hepatic lipid synthesis could then be correlated with the magnitude of the birds' response to oestrogen treatment.

CHAPTER 3

THE INCORPORATION OF [1-^{14}C] ACETATE, $^{3}H_2O$ AND [9,10-^{3}H] PALMITATE INTO LIPIDS BY LIVER SLICES FROM CONTROL AND OESTROGEN-TREATED MALE CHICKS

INTRODUCTION

1. Biochemical techniques for studying metabolic processes

A variety of techniques have been developed to study metabolic pathways both in the intact living animal and in isolated tissue preparations. The use of a combination of *in vitro* and *in vivo* techniques is important, since a detailed knowledge of a metabolic process cannot be obtained by the use of either type of technique alone, and each has its advantages and limitations. Possible mechanisms and enzyme systems can be best investigated with purified tissue preparations, but interpretation of the results of experiments of this kind and extrapolation to the situation existing in the living animal involve a certain amount of speculation. The metabolic processes that take place within an animal are interrelated and are under complex homeostatic control, and this relationship is obviously disturbed when tissues are isolated. Consequently, although *in vitro* techniques are invaluable for determining possible metabolic mechanisms, only *in vivo* studies can provide information about the processes that actually occur in the living animal. In this report, various techniques that have been used in the study of liver metabolism will be reviewed, since this is the organ under investigation in the present study.

Tissue preparations used in *in vitro* studies vary considerably in their degree of deviation from the natural state. Preparations commonly used in the study of liver metabolism range from the intact perfused organ to tissue slices and fragments, cell suspensions, homogenates composed of broken cells, preparations of isolated organelles and pure enzyme preparations. The least disrupted preparation involves the isolation of the intact liver, which can be perfused via the blood vessels with isotonic media containing oxygen and nutrients that

are able to sustain the metabolic activity of the organ [121, 243]. Using such preparations it is possible to study normal processes occurring in the liver, and the responses of the liver to a variety of agents that can be added to the perfusion medium. The advantages associated with this *in vitro* technique are that the cells remain intact and retain their natural positions and cell to cell contact within the organ. Nevertheless, the perfusion of the isolated liver is not an ideal reproduction of the situation existing *in vivo* since, as with other isolated tissue preparations, humoral and nervous influences are lacking.

Another isolated tissue preparation which retains the cell to cell interactions is the liver slice [30, 71, 113, 236, 238, 370]. The advantage of using slices immersed in isotonic media containing necessary nutrients and oxygen, rather than the perfused liver, is that a variety of different experiments can be performed with tissue from the same liver, and both control and experimental determinations can often be carried out using samples from the same organ. The preparation of slices is also quick and easy once the technique of cutting is mastered. The disadvantages of using liver slices include the variability in surface area and thickness of the slices, which affect the diffusion of oxygen and substrates into the cells and hence can limit the metabolic activity of the cells. The process of diffusion is better in the perfused liver, since the distance between the capillary wall and the liver cells is small compared with the distance between the cells in the centre of a liver slice and the medium in which it is suspended. Problems associated with liver slices also include the presence of cut cells on the surface and 'shocked' cells in the interior. Damage to cells in liver slices has been indicated by the leakage of small molecular weight constituents such as adenine nucleotides and, in extreme cases, intracellular enzymes into the medium.

The problem of diffusion has been partly overcome by the use of 'liver snips' [371], which have been shown to be less damaged and more active than liver slices, and to yield results with greater reproducibility. Liver snips are prepared by manual snipping, and this has been reported to cause damage to less cells than is customary with a tissue slicer. Snips are also much smaller than liver slices and, therefore, samples taken for experiments are more homogeneous and diffusion distances are reduced. The preparation of snips is much easier than that of slices, which requires considerable practice before reasonable preparations can be produced. Also, the preparation of slices from foetal, infant, fatty and other small or fragile livers can be extremely difficult, and the preparation of snips is more practical in such cases.

The problem of diffusion of substrates posed by the use of liver slices and liver snips has been solved by the use of isolated hepatocytes, prepared by enzymic digestion of the proteins and mucopolysaccharides holding cells together, which can be freely suspended in media containing nutrients and oxygen [276, 372 - 375]. This preparation has the advantage that samples taken for experimentation are more homogeneous than is the case with slices and snips, which contain a high proportion of ruptured cells and also connective tissue, blood vessels, and other cell types in addition to liver parenchymal cells. A disadvantage associated with the use of this type of tissue preparation, when compared with liver slices and liver snips, is that hepatocytes can take rather longer to prepare, and ageing of the cells may complicate experimentation. In addition, cell to cell contact is lost, and the preparation of hepatocytes involves enzymic digestion at cell surfaces, which may affect the plasma membranes and receptors situated at the surface of the hepatocytes [373]. Nevertheless, isolated hepatocytes can be advantageous, since they avoid the complex interactions

imposed on cells *in vivo* and, therefore, are often more convenient for experimentation.

A major problem with the use of the tissue preparations described above is that they are limited to acute studies of metabolism because they are viable for only a few hours. The development of cells in primary culture that are able to retain their differentiated functions are, therefore, more useful in extended studies [372, 374 - 376], although with longer periods the degree of deviation from the natural state becomes greater.

The use of tissue homogenates, isolated organelles and purified enzyme preparations has proved extremely useful for the elucidation of possible metabolic pathways and enzyme systems. The separation of organelles by density gradient centrifugation allows the isolation, in good yield and purity, of most cellular components. However, the transport of ions and compounds into isolated organelles may not be the same as *in vivo*, thus giving rise to an artificial situation. In addition, preparations of a particular organelle from a tissue may not necessarily be homogeneous, and may include organelles from different regions of the tissue which differ biochemically. In tissue homogenates and purified enzyme preparations, substrates can be presented directly to enzyme molecules without any problems of diffusion into cells, since the cell membranes are disrupted. In addition, homogenates of broken cells can be 'purified' to varying degrees. The disadvantages of using these preparations become evident when one tries to extrapolate from the results of experiments using such preparations to the situation existing in the living cell and in the animal as a whole. The process of cell disruption breaks up the structure of the cell, causing the mixing of components that might be separated in different cellular compartments in the living state. Hence, enzymes may be brought into contact with

a variety of activators, inhibitors, substrates and lysosomal enzymes from which they would be separated in the intact living cell. Therefore, enzymes assayed in homogenates and subcellular preparations may be inhibited or activated compared with the activities they express in the living cell. Also, the addition of various cofactors and substrates to homogenates and purified enzyme preparations may give a false impression of the activities of enzymes in the intact cell, where the concentrations of these components may be limited, or greater due to concentration in cellular compartments. Such studies, therefore, can provide information about the relative quantities of enzymes present in cells, but give no indication of the actual activities and their relative importance in the living state.

One of the most useful in vivo experimental techniques for the study of metabolic pathways involves the employment of radioactive tracers [20, 25, 67, 114, 252, 377]. Labelled substances can be administered orally or by injection into the bloodstream or any part of the body, and at specified times after administration body fluids can be withdrawn or tissues can be isolated and the fate of the radioisotope can be determined. This technique is particularly advantageous since natural conditions can be maintained in the living animal, and metabolic pathways can be elucidated by isolating labelled intermediates at different times after administration of the radioactive substrate. The major problem arising from the use of this technique is the interpretation of the results obtained. For example, the presence of a labelled product at a particular site in the body does not necessarily imply that the product is synthesized there, since the product may be manufactured elsewhere in the body and be translocated via the circulation. Similarly, even though the product may be synthesized in a particular tissue or organ, the amount of product isolated from that

site may not be an accurate measure of the synthetic capacity at that location, since the product may be translocated from one organ or tissue to another via the bloodstream.

2. Hepatic lipid metabolism of the domestic fowl

In the present study, liver slices were used to investigate lipid metabolism in the livers of male chicks after they had been treated with 17 β-oestradiol. In Chapter 2, it was shown that such treatment causes elevations of plasma triacylglycerol levels, which are associated predominantly with increases in plasma VLDL [113 - 115, 117, 118, 236, 242]. Hepatic lipid metabolism in oestrogen-treated birds has received considerable attention, since the liver is considered to be the major site of lipogenesis in avian species [20 - 27], and the domestic fowl does not develop hypertriacylglycerolaemia in response to oestrogens after functional hepatectomy [122]. Extrapolation from these studies to the changes that occur in the hen approaching lay have also been attempted, since both situations involve similar changes in blood and liver lipids and proteins, and it is reasonable to believe that similar metabolic processes are involved [56, 58, 81, 82, 115, 242].

A variety of *in vivo* and *in vitro* techniques have been employed to investigate hepatic lipid metabolism in the chick, the adult cockerel, male and immature female birds after oestrogen treatment, and in the laying hen, but some of the results have been confusing and conflicting, and we do not have a clear picture of the lipogenic changes that occur in birds after oestrogenization or at the onset of lay in the hen. Several investigators have employed liver slices, incubated with precursors of complex lipids, in order to detect increases in hepatic lipid synthesis and to determine the time courses of such changes. Various labelled non-lipid precursors and fatty acids have been used in

these liver slice studies, and the results of some of these investigations will be outlined below.

A number of research workers have tried to elucidate the differences in lipid metabolism that must occur between the immature female bird and the laying female, by virtue of the greater demand for lipid existing in the laying hen associated with egg production. Duncan (370) measured the incorporation of [1-^{14}C] glucose, [6-^{14}C] glucose and [U-^{14}C] glucose into total lipids by liver slices from 10 to 13-week old immature female fowl and from 26 to 32-week old laying hens, and observed higher incorporations by slices from the immature birds than from the laying birds when expressed on a wet tissue weight basis. However, the differences did not attain statistical significance at the 5% level using Student's 't' test, and when expressed on a cellular basis this difference was almost eliminated. Similar results have been reported by Weiss et al. (237) and Leveille (239), who studied the incorporation of [1-^{14}C] acetate into lipids by liver slices from chicks and hens. Weiss et al. (237) observed levels of incorporation of [1-^{14}C] acetate into lipids by mature hen liver slices which were about 2% of the levels observed by Leveille (33) for chicks. This observation led Leveille (239) to study the situation in the hen and chick simultaneously, and he showed that liver slices from male chicks and mature hens had similar capacities for fatty acid synthesis from [1-^{14}C] acetate.

Duncan (238) reported that liver slices from 9 to 15-week old immature female domestic fowl incorporated more [1-^{14}C] acetate into fatty acids than did those of laying hens of 26 to 32 weeks of age, whereas no differences occurred in the conversion of [U-^{14}C] glycerol, [2-^{14}C] glutamate, [5-^{14}C] glutamate and [3-^{14}C] aspartate to fatty acids. When the incorporation results for liver slices from immature female birds were compared with those for mature male birds (35 to 40

weeks old), it was found that the immature female birds converted more [1-^{14}C] acetate, [2-^{14}C] glutamate and [5-^{14}C] glutamate into fatty acids, whilst the amounts of fatty acid synthesized from these substrates by liver slices from cockerels and laying hens were not significantly different. Similarly, Leveille et al. (20) studied lipogenesis of the domestic fowl in vivo using [U-^{14}C] glucose and [1-^{14}C] acetate, and observed rates of hepatic lipogenesis in the chick that were similar to those reported previously in the hen (377), but considerably higher than those observed in the adult cockerel (377). These observations suggest that hepatic fatty acid synthesis does not increase as the hen comes into lay. This conclusion is rather unexpected in view of the massive amounts of lipid that are produced by the laying hen, and considering the fact that the liver of the domestic fowl is extremely important in the de novo synthesis of fatty acids (20 - 22, 30, 31).

The conflicting results of some of the liver slice experiments presented above are of particular interest when viewed in conjunction with the results of lipogenic enzyme activity studies. For example, Pearce (378) showed that the specific activities of ATP citrate lyase (EC 4.1.3.8) and 'malic' enzyme (EC 1.1.1.40)* were similar in the livers of laying hens and of 4 and 7-week old pullets. This is in agreement with the observations of Leveille (239) who showed, by studying the incorporation of [1-^{14}C] acetate into lipids by liver slices, that the rate of hepatic lipogenesis was similar in the chick and the mature laying hen. Pearce (378) showed that the hepatic activities of ATP citrate lyase and 'malic' enzyme in the female bird decreased sharply between 7 and 10 weeks of age, remained low until at least 22 weeks of age, and increased again at sexual maturity. Similar observations were made by Raheja et al. (359) for hepatic 'malic' enzyme activity in the cockerel during development, although in the cockerel the level of

* malate dehydrogenase (decarboxylating ; NADP$^+$)

activity of this enzyme remains low throughout adulthood. In accordance with these results, Pearce [125] and Pearce & Brown [246] observed greater hepatic specific activities of ATP citrate lyase and 'malic' enzyme in the laying hen than in the cockerel or non-laying mature female, while the hepatic enzyme specific activity levels observed in non-laying females and cockerels were similar. The general picture, therefore, resulting from these studies is that lipogenesis is high in the livers of young chicks, but decreases to a lower level with age. In the cockerel the rate of lipogenesis remains low, but at sexual maturity in the hen lipogenesis increases once again.

Several workers have studied liver slices from oestrogen-treated birds in attempts to elucidate the mechanism of increased lipogenesis in these birds. Duncan [238] treated immature 9 to 15-week old female chickens with 1 mg 17 β-oestradiol/bird/day for 10 days, and observed increased fatty acid synthesis from [1-^{14}C] acetate, but not from [U-^{14}C] glucose, [2-^{14}C] glutamate or [5-^{14}C] glutamate, by liver slices from these birds compared with those from control pullets. Liver slices from control immature female birds were found to incorporate more [1-^{14}C] acetate into fatty acids than did those from laying hens, and therefore, treatment with oestrogen increased the fatty acid synthetic capacity from [1-^{14}C] acetate shown by the immature female bird still further. This stimulation of hepatic fatty acid synthesis by exogenous oestrogen was, therefore, not comparable with the normal physiological situation in the laying hen, in which metabolism is under the influence of endogenous oestrogens. A similar increase in the incorporation of [1-^{14}C] acetate into lipids by liver slices from oestrogen-treated birds was shown by Kudzma et al. [113]. These investigators treated 5-day old male and female chicks with 0·1 mg diethylstilbestrol daily for 18 days, and 24 hours after the last injection chicks were

sacrificed and liver slices were prepared. Incorporation of [^{14}C] acetate into total lipids by liver slices was found to be approximately 3-fold greater for oestrogen-treated chicks than for control chicks. This increase in [^{14}C] acetate incorporation after oestrogen treatment became a 4·5-fold increase when incorporation into triacylglycerol was presented in isolation. In addition, Kudzma et al. (114) reported that treatment of 1-week old chicks with a daily injection of 0·5 mg diethylstilbestrol for 8 days increased the in vivo incorporation of [1-^{14}C] acetate or [U-^{14}C] glucose into blood and liver lipids, predominantly the triacylglycerol fraction of VLDL. Studies on young turkey hens, with body weights of 10 kg, have also shown that oestrogen treatment (a 25 mg dose of 17 β-oestradiol followed by a 75 mg dose 1 week later) causes an increase in de novo lipogenesis from [1-^{14}C] acetate in liver slices (251).

The activities of ATP citrate lyase and 'malic' enzyme in the livers of oestrogen-treated pullets have been investigated and have yielded somewhat ambiguous results. Pearce & Brown (246) treated immature pullets with oestradiol dipropionate in the form of a subcutaneous pellet for a week, and observed no significant differences in the hepatic specific activities of ATP citrate lyase and 'malic' enzyme between oestrogen-treated and control birds. However, Balnave & Pearce (166, 256) treated 4-week old pullets with injections of oestradiol dipropionate (0·5 - 4 mg oestradiol dipropionate/injection) on alternate days, and observed maximum hepatic specific activities of ATP citrate lyase and 'malic' enzyme on the 2nd day, after just one dose of 2 mg and 1 mg oestradiol dipropionate/bird respectively. The specific activities of these enzymes were decreased, and often depressed below control values, after longer times of oestrogen administration and/or after treatment with higher oestrogen doses. It would, therefore, appear

that short-term oestrogen treatment ($\leq$ 2 days) at a carefully selected dose is more indicative of physiological effects and the situation in the laying hen than are high oestrogen doses and long-term treatment.

One of the major problems associated with the use of radioisotopically-labelled non-lipid precursors such as glucose and acetate for studying lipid metabolism, both *in vivo* and *in vitro*, is that interpretation of the incorporation results remains speculative unless the cellular pool sizes and specific radioactivities of the immediate precursors of the products are known. There is always the possibility that ^{14}C-labelled substrates underestimate *de novo* lipogenesis, because the rate of entry of the substrates into cells and their metabolism to acetyl-CoA may be rate-limiting and because the specific radioactivity of the substrate, or of acetyl-CoA generated from it, is diluted within the cell. Therefore, differences between oestrogen-treated and control birds in the hepatic incorporation of labelled substrates such as acetate into lipids may be obscured if the cellular pool sizes of acetyl-CoA differ in these birds. For example, the absence of increased incorporation of labelled acetate into fatty acids by oestrogenized chick liver may merely reflect an oestrogen-induced increase in the cellular acetyl-CoA pool size, resulting in the dilution of labelled acetyl-CoA. Conversely, increased incorporation may merely reflect a decrease in acetyl-CoA pool size and concentration of labelled acetyl-CoA. In addition, there may be differences in the facility to activate acetate to acetyl-CoA, and differences in permeability of the tissue to acetate.

A means of avoiding problems of precursor pool sizes and specific radioactivities is to use $^{2}H_2O$ or $^{3}H_2O$ as the labelled substrate for *de novo* lipogenesis (22, 30, 46, 139, 160, 252, 276, 365, 375, 379 - 381), since the concentration of water in the incubation medium or body fluids and in the cells is very high, and the specific radioactivity of the

hydrogen being incorporated into lipids is relatively constant despite large variations in the rates of lipid synthesis and other metabolic processes [382]. Hydrogen from water is incorporated into fatty acids and cholesterol during their syntheses, but there is no incorporation of isotopic label into pre-formed lipids [383, 384]. Hydrogen from water is incorporated into lipids regardless of the form of the carbon substrate and, therefore, provides an accurate measure of total de novo lipogenesis, as opposed to incorporation studies using a ^{14}C-labelled substrate which measure fatty acid synthesis from that substrate alone. Fatty acids synthesized in the presence of $^{2}H_2O$ or $^{3}H_2O$ have isotopic label incorporated into stable linkages at both odd- and even-numbered carbon atoms [383, 384]. A disadvantage of using $^{2}H_2O$ or $^{3}H_2O$ for the measurement of metabolic processes is that the radioisotopes have a larger mass than protium and may, therefore, react more slowly, and the rate of incorporation may not be a true reflection of the rate of the process under investigation. However, it is reasonable to assume that such an isotope effect would be the same in all animals and cells and, therefore, comparisons of incorporation results would be valid.

Dashti et al. [252, 365] have applied the use of $^{3}H_2O$ to the measurement of de novo lipogenesis in oestrogen-treated birds, and administered $^{3}H_2O$ in vivo to immature male turkeys after treatment with diethylstilbestrol. These workers injected 19-day old male turkeys with diethylstilbestrol (40 mg/kg body wt.) in corn oil, or with corn oil only, and killed the birds immediately or 24, 48 or 72 hours later [252]. One hour before death, birds were injected intraperitoneally with 20 mCi $^{3}H_2O$, and the incorporation of $^{3}H_2O$ into total liver and plasma lipids was determined. The incorporation of $^{3}H_2O$ into total lipid was approximately 3·9-fold greater for oestrogenized birds than for control birds at 24 hours after hormone and/or corn oil injection,

and this increased to a 5·5-fold difference at 48 hours. The increased incorporation of tritium from $^{3}H_2O$ was mainly into liver triacylglycerols which were secreted into the blood predominantly as VLDL.

The controversial results that have been obtained in the studies of de novo fatty acid synthesis in chicks, cockerels, hens, and oestrogenized male and immature female domestic fowl have occasionally suggested that at sexual maturity in the hen and after oestrogenization of birds the liver may not develop an enhanced capacity to synthesize fatty acids. Instead, fatty acids may, theoretically, be manufactured at extra-hepatic sites or be mobilized from adipose tissue, and may be transported via the circulation to support an enhanced production of complex lipids by the liver. This could possibly explain the lower level of fatty acid synthesis from [1-^{14}C] acetate registered for the livers of laying hens than for the livers of immature female birds (237, 238, 370), since hepatic fatty acid synthesis may be inhibited in the hen by fatty acids arriving from extra-hepatic sources. This process may also be operative in the oestrogenized bird. The mobilization of fatty acids from adipose tissue would be a rather wasteful way of obtaining fatty acids for hepatic lipogenesis, since the liver of avian species has a high capacity for de novo fatty acid synthesis, whereas that of adipose tissue is low. Consequently, the majority of the fatty acids in adipose tissue are of hepatic origin.

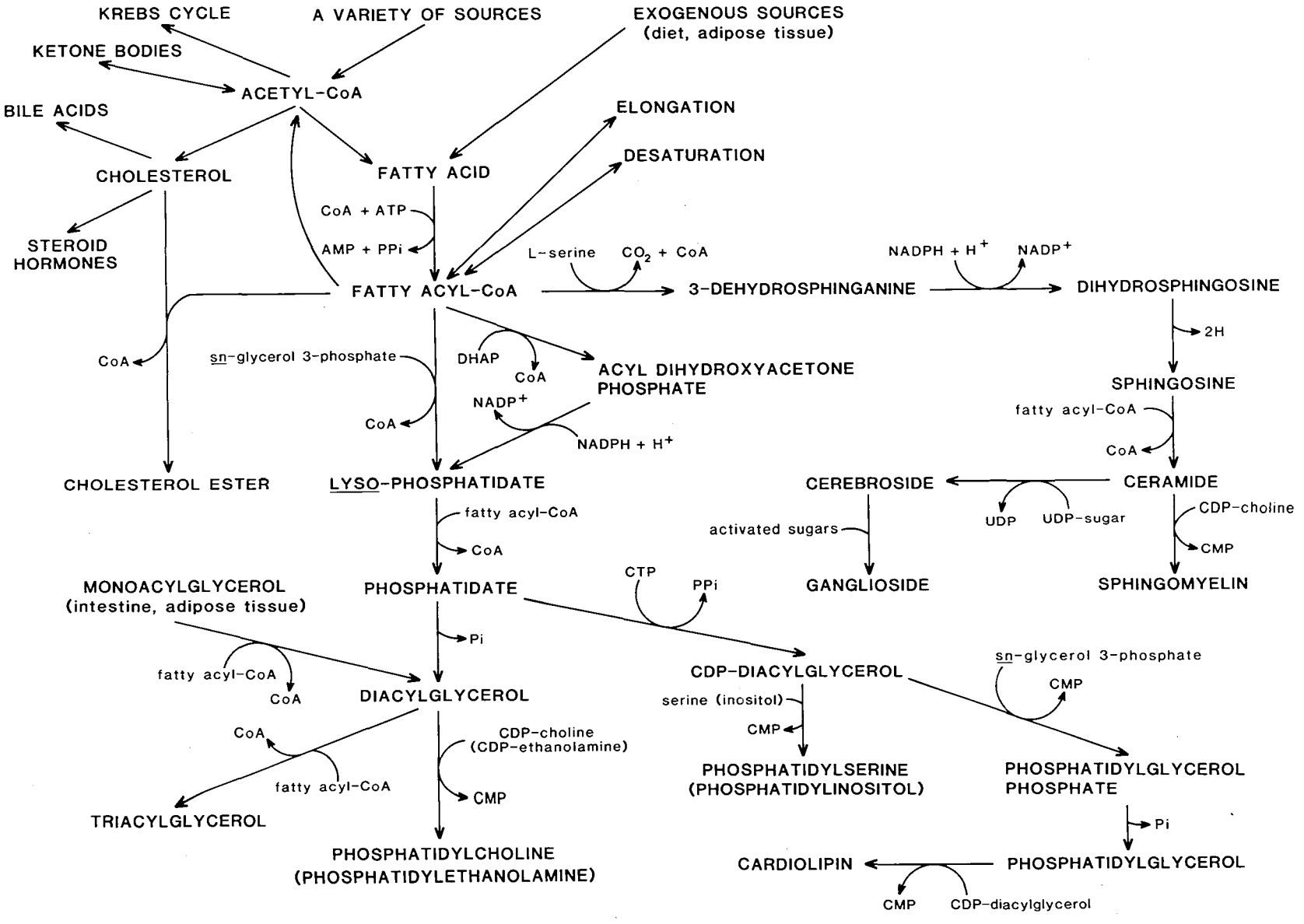
At the onset of lay in the hen, the amounts of complex lipids and free fatty acids in the plasma increase simultaneously (57, 234). Similar increases are observed in oestrogen-treated birds (113, 164, 235), although it has been suggested that the increase in plasma free fatty acids does not occur until after the hypertriacylglycerolaemia develops, implying that the fatty acids may be of hepatic origin (113). Pageaux et al. (235) have demonstrated a significant decrease in serum

free fatty acids 1 hour after the administration of oestradiol benzoate (0·2 mg/kg body wt.) to 16-day old female quail, although this was followed by increased concentrations over the following 23 hours. This implies that, at least in the early stages after oestrogen treatment, the removal of fatty acids from the bloodstream may be stimulated. Oestrogen treatment of Xenopus laevis has also been shown to produce an increase in the level of free fatty acids in the plasma (60). The origins of the plasma free fatty acids at the various times after oestrogen treatment of male and immature female domestic fowl, and during the development of sexual maturity in the hen, have not been determined. Consequently, it is not known if they are in a state of transport to the liver from extra-hepatic sites such as adipose tissue, if they have been released from circulating VLDL after lipolysis by lipoprotein lipase in peripheral tissues, or if they emanate from the liver as a result of overproduction.

Several workers have demonstrated that the livers of the laying hen and oestrogenized domestic fowl have an increased capacity to incorporate pre-formed fatty acids into complex lipids (114, 236, 238). Pre-formed fatty acids and fatty acids synthesized de novo may be incorporated into complex lipids via the pathways illustrated in Fig. 10. Hawkins & Heald (236) showed that, on a dry weight basis, liver slices of the laying hen incorporated almost twice as much [1-^{14}C] palmitate into neutral lipids than slices from immature female birds. On a cellular basis, using DNA as a standard for comparison, liver slices from the laying hen incorporated approximately 2·5-fold more [1-^{14}C] palmitate into neutral lipids than did slices from immature female birds. Since the intracellular free fatty acid pool in the liver of the laying bird was shown to be twice that in the liver of the immature bird, liver cells of the laying hen would appear to possess a 5-fold

FIGURE 10

Schematic representation of the fates of fatty acids in the cell


KREBS CYCLE
A VARIETY OF SOURCES
EXOGENOUS SOURCES
(diet, adipose tissue)
KETONE BODIES
ACETYL-CoA
BILE ACIDS
ELONGATION
DESATURATION
CHOLESTEROL
FATTY ACID
CoA + ATP
AMP + PPi
STEROID
HORMONES
L-serine
CO_2 + CoA
NADPH + H^+
$NADP^+$
FATTY ACYL-CoA
3-DEHYDROSPHINGANINE
DIHYDROSPHINGOSINE
2H
CoA
sn-glycerol 3-phosphate
DHAP
CoA
ACYL DIHYDROXYACETONE
PHOSPHATE
SPHINGOSINE
fatty acyl-CoA
CoA
$NADP^+$
CoA
NADPH + H^+
CHOLESTEROL ESTER
LYSO-PHOSPHATIDATE
CEREBROSIDE
CERAMIDE
UDP
UDP-sugar
CDP-choline
fatty acyl-CoA
activated sugars
CoA
CMP
MONOACYLGLYCEROL
(intestine, adipose tissue)
PHOSPHATIDATE
CTP
PPi
GANGLIOSIDE
SPHINGOMYELIN
Pi
fatty acyl-CoA
CoA
DIACYLGLYCEROL
CDP-DIACYLGLYCEROL
sn-glycerol 3-phosphate
CMP
serine (inositol)
CMP
CoA
CDP-choline
(CDP-ethanolamine)
fatty acyl-CoA
CMP
PHOSPHATIDYLSERINE
(PHOSPHATIDYLINOSITOL)
PHOSPHATIDYLGLYCEROL
PHOSPHATE
TRIACYLGLYCEROL
Pi
PHOSPHATIDYLCHOLINE
(PHOSPHATIDYLETHANOLAMINE)
CARDIOLIPIN
PHOSPHATIDYLGLYCEROL
CMP
CDP-diacylglycerol

greater capacity to incorporate fatty acids into neutral lipids than do liver cells of the immature female domestic fowl. Similarly, Duncan (238) demonstrated that liver slices from laying birds incorporated more [U-^{14}C] glycerol into the glycerol moiety of triacylglycerols than did liver slices from immature female birds. In addition, there was no difference between laying and immature birds in the incorporation of [U-^{14}C] glycerol, [2-^{14}C] glutamate, [5-^{14}C] glutamate and [3-^{14}C] aspartate into fatty acids by liver slices, and the incorporation of [1-^{14}C] acetate into fatty acids was lower in liver slices from the laying hen than in those from the immature female bird. These results suggest that additional fatty acids required for hepatic triacylglycerol formation during egg production might be derived from extrahepatic sources. Kudzma et al. (114) showed that there was an increased incorporation of plasma free fatty acids into triacylglycerols by the livers of oestrogenized chicks compared with control chicks. These workers injected chicks (850 - 1000 g body wt.) daily, for 4 days, with a dose of diethylstilbestrol (2 mg/day/bird) in sesame oil or with sesame oil only, and 24 hours after the last injection the birds were killed. Birds were given 25 µCi of plasma-bound sodium [9,10-^{3}H] palmitate intravenously 5 - 7 minutes before death, and immediately after death the livers and plasma samples were rapidly removed, and the fraction of injected label converted to triacylglycerols was determined. The plasma free fatty acid pool size was increased 5-fold and the fractional conversion of [9,10-^{3}H] palmitate increased 2-fold after oestrogen treatment, and hence it was calculated that the livers of oestrogenized chicks converted 10-fold more free fatty acid to triacylglycerol than did the livers of control chicks. It would appear, therefore, that the livers of laying hens and oestrogenized male and immature female domestic fowl possess an enhanced capacity to incor-

porate fatty acids, whether they be of hepatic or extra-hepatic origin, into neutral lipids, predominantly into triacylglycerols.

The aim of the present work was to study the relative importance of non-lipid precursors and pre-formed fatty acids in the biosynthesis of glycerolipid in the liver of the oestrogen-treated male chick at various times after hormone administration. The incorporation of [1-^{14}C] acetate or $^{3}H_2O$ into glycerolipid by liver slices from oestrogen-treated and control chicks was taken as a measure of lipogenesis via the de novo biosynthesis of fatty acids. On the other hand, the incorporation of [9,10-^{3}H] palmitate into glycerolipid by liver slices was taken as a measure of lipogenesis from pre-formed, exogenous fatty acid. It was hoped that this work might help to resolve some of the anomalies that have appeared in the literature concerning the effect of oestrogen on lipid metabolism in avian liver.

METHODS

1. [1-^{14}C] Acetate incorporation studies

Preparation and storage of solutions

A modified Krebs-Ringer bicarbonate buffer, pH 7·4, was used in these studies (385, 386). The compositions of the buffers employed were as follows:-

Component	Volume (ml)		
	Buffer A	Buffer B	Buffer C
4·5% (w/v) NaCl	20	20	18
5·75% (w/v) KCl	4	4	4
6·1% (w/v) $CaCl_2$	0·6	0·6	0·6
3·82% (w/v) $MgSO_4.7H_2O$	1	1	1
2·11% (w/v) KH_2PO_4	1	1	1
1·3% (w/v) $NaHCO_3$	25	25	25
D-glucose (0·572 M)	2·5	—	—
$+H_2O$ to total volume (ml) of:-	130	130	117

Buffers were made up fresh each day by mixing of the components. Solutions of $NaHCO_3$ were made up fresh just before use. The other component solutions were stored at 4°C, and fresh solutions were prepared every 7 - 14 days. The solutions were discarded sooner if there was obvious bacterial contamination.

All buffers were gassed with O_2:CO_2 (95%:5%) at 40°C for 30 - 40 minutes before use, and the pH of each was adjusted to 7·4 with HCl after gassing. Most experiments ran for several hours and the buffers were gassed periodically throughout.

Ice-cold buffer A was used for rinsing livers and storing liver cubes and slices before incubation. Prior to incubation, the liver slices were rinsed free of extraneous glucose by immersion in ice-cold buffer B. Buffer C was the buffer in which liver slices were incubated, and was more concentrated than buffers A and B to allow for dilution following additions to incubations. Buffer C also contained less NaCl to compensate for Na^+ ions added to incubations as sodium acetate.

The buffer composition adopted is very close to that of the avian saline described by Sturkie [387]. A comparison of mammalian Krebs-Ringer bicarbonate buffer [386], the buffers used in the present study, and the avian saline of Sturkie [387] is as follows:-

Component	Concentration (mM)		
	Mammalian Ringer (Dawson [386])	Buffers A and B	Avian saline (Sturkie [387])
NaCl	118·47	118·47	116·36
KCl	4·75	23·73	23·20
$CaCl_2$	2·54	2·54	5·77
$MgSO_4 \cdot 7H_2O$	1·19	1·19	1·01
KH_2PO_4	1·19	1·19	—
$NaHCO_3$	25	29·76	29·16

The ionic concentrations of the 3 types of buffer are shown below:-

Ionic component	Concentration (mequiv./litre)		
	Mammalian Ringer (Dawson [386])	Buffers A and B	Avian saline (Sturkie [387])
Na^+	143·47	148·23	145·52
K^+	5·94	24·92	23·2
Ca^{2+}	5·08	5·08	11·54
Mg^{2+}	2·38	2·38	2·02
Cl^-	128·3	147·28	151·1
$H_2PO_4^-$	1·19	1·19	—
SO_4^{2-}	2·38	2·38	2·02
HCO_3^-	25	29·76	29·16

The buffer used in the present study differs from that recommended by Sturkie [387] in that it contains approximately half the concentration of $CaCl_2$, and KH_2PO_4 is included at a concentration of 1·19 mM. The avian ringers differ from the mammalian ringer primarily by the inclusion of 5 times the concentration of KCl. This is to accommodate the fact that avian blood contains more potassium than does mammalian blood [388], and to try to maintain normal intracellular potassium concentrations [389]. The calcium and chloride concentrations of Sturkie's avian saline are high compared with the mammalian ringer. These concentrations were reduced towards those of the mammalian ringer in the buffer adopted for this study. The inclusion of less calcium reduced the possibility of precipitation on mixing of the components.

Liver slice preparation and incubation procedures

Chicks were killed by decapitation and livers were rapidly removed, weighed, and immersed in ice-cold avian bicarbonate buffer containing 11 mM-glucose (buffer A). Portions of liver were cut into small cubes (3 - 4 mm in dimension) and slices were prepared from these liver cubes with a Stadie-Riggs hand microtome. Weighed pieces of liver were frozen immediately for future DNA determinations. Liver cubes and slices were kept in ice-cold buffer A. Prior to incubation, slices were rinsed in avian bicarbonate buffer without glucose (buffer B), blotted on filter paper and weighed by means of a torsion balance. Incubations were routinely started within 10 minutes of killing the bird. Triplicate incubations were performed for each liver. The last incubation was normally started within 16 - 18 minutes of the death of the bird.

Incubations were carried out in 25 ml Erlenmeyer flasks fitted with rubber 'Suba-seal' stoppers through which gassing needles could be inserted. Flasks contained 2·7 ml buffer C and 0·3 ml sodium [1-^{14}C]

acetate solution ($1\frac{1}{2}$ - 3 μCi/flask). Preliminary experiments were performed to determine the optimum acetate concentration (10 mM) and a suitable incubation time (1 hour). Flasks were incubated at 40°C in a shaking water bath. After a 10 minute pre-incubation period, during which flasks were gassed with O_2:CO_2 (95%:5%), liver slices (150 ± 10 mg, wet wt.) were added to each flask and the incubation time began. Flasks were gassed with O_2:CO_2 (95%:5%) for the first 10 minutes of the incubation time, after which the gassing needles were withdrawn.

Reactions were stopped by the addition of either 0·24 ml conc. HCl (11·8 M) or 11·25 ml chloroform/methanol (1:2, v/v). The stoppered flasks were immediately packed in ice, prior to storage at -20°C until lipid extractions could be carried out. Lipid extractions were routinely performed within a week.

Lipid extraction procedures

In preparation for lipid extraction, the liver slices (150 ± 10 mg, wet wt.) and incubation medium (approx. 3 ml) from each flask were homogenized in 11·25 ml chloroform/methanol (1:2, v/v) and 0·24 ml conc. HCl with the aid of a Teflon-glass homogenizer. The tissue was disrupted with 10 'up and down' strokes of the rotating pestle. The homogenates were transferred to stoppered extraction tubes and lipid was extracted by the method of Hajra et al. (390). Chloroform (3·75 ml) was added to each tube and the contents were mixed thoroughly for 30 seconds, and then 3·75 ml 2 M-KCl in 0·2 M-H_3PO_4 were added and the contents were mixed for 30 seconds again. Subsequently, the tubes were left on ice until the phases separated. The upper aqueous phase from each extraction was removed, and the bottom phase was washed thrice with 12·6 ml of 'synthetic top phase' containing 5 mM-sodium acetate as carrier.

Aliquots of the lipid extracts were evaporated to dryness under nitrogen and were dissolved in 10 ml of xylene containing 4 g PPO and

0·1 g PoPoP/litre. Samples of the stock radioisotope solutions were made up to 1 ml with water and were mixed with 10 ml of xylene/Triton X-100 (2:1, v/v) containing 5·5 g PPO and 0·1 g PoPoP/litre to determine the amount of radioactivity added to each incubation. Radioactivity was measured by liquid scintillation counting in a Nuclear Enterprises 8312 Automatic Spectrometer and a Packard 300C Tri-Carb Liquid Scintillation Counter.

Analysis of extracted lipid from liver slices by thin layer chromatography

In some cases, samples of the lipid extracts were taken for analysis by thin layer chromatography (t.l.c.). An aliquot (1·5 - 2 ml) of lipid extract was taken from each flask extraction, and aliquots from the same bird were pooled. The lipid extract was dried under nitrogen, dissolved in a small volume of chloroform, and aliquots were applied as 2 cm 'streaks' to activated plates of Kieselgel 60H. Aliquots were also taken, dried under nitrogen, and dissolved in 10 ml of xylene containing 4 g PPO and 0·1 g PoPoP/litre, for estimation of the amount of radioactivity added to the plate.

The solvent system employed to resolve lipid classes was light petroleum (b.p. 40 - 60°C)/diethyl ether/acetic acid (60:40:1, by vol.). After separation, the lipid classes were visualized by exposing the dried plates to iodine vapour, followed by marking of the stained areas and decolourization in a stream of warm air. Authentic neutral lipid standards and oleic acid were run on the same plates to establish the identities of the lipid spots. The lipid classes separated by this solvent system, and the R_F values obtained for these classes, are presented in Table 5.

The lipid spots and the intermediate areas were scraped off the plates into scintillation vials and 10 ml of xylene containing 4 g PPO

TABLE 5

Lipid classes and their R_F values obtained after t.l.c. analysis of extracted lipid from liver slices

Lipid class	R_F (mean ± S.E.M.)
Triacylglycerol	0·78 ± 0·005 (145)
Free fatty acid	0·44 ± 0·004 (118)
1,3-diacylglycerol	0·33 ± 0·005 (58)
1,2-diacylglycerol + cholesterol	0·26 ± 0·004 (58)
Monoacylglycerol	0·04 ± 0·004 (4)
Total phospholipid	Origin

The solvent system employed to resolve the lipid classes was light petroleum (b.p. 40 - 60°C)/diethyl ether/acetic acid (60:40:1, by vol.).

The numbers in parentheses represent the number of values used in calculating the mean and S.E.M. in each case.

and 0·1 g PoPoP/litre were added to each. The vials were shaken to solubilize the lipid, and radioactivity was measured by liquid scintillation counting.

Saponification of lipids in lipid extracts from liver slices

Samples (100 µl) of the Hajra lipid extracts were evaporated to dryness under nitrogen in extraction tubes. NaOH (1 ml of 2 M in 50% (v/v) ethanol) was added to the lipid and the stoppered tubes were heated at 100°C for 2 hours. After cooling, water (3 ml) was added. Non-saponifiable lipids were removed by 2 successive extractions with 3 ml light petroleum (b.p. 40 - 60°C). The pH of the aqueous phase was then adjusted to 1 - 2 with H_2SO_4. Fatty acids derived from complex lipids were removed by 3 successive extractions with 3 ml light petroleum (b.p. 40 - 60°C). The light petroleum extracts were dried under nitrogen. Scintillation fluid (10 ml of xylene containing 4 g PPO and 0·1 g PoPoP/litre) was added to each dried extract, and radioactivity was determined by liquid scintillation counting.

2. ^3H_2O incorporation studies

Procedures were as described for the [1-^{14}C] acetate incorporation studies with minor modifications. Buffer C was made up as follows, to compensate for Na^+ ions added to incubations as sodium acetate:-

Component	Volume (ml)
4·5% (w/v) NaCl	19
5·75% (w/v) KCl	4
6·1% (w/v) $CaCl_2$	0·6
3·82% (w/v) $MgSO_4.7H_2O$	1
2·11% (w/v) KH_2PO_4	1
1·3% (w/v) $NaHCO_3$	25
H_2O	66·4
Total volume:-	117

Incubation flasks contained 2·7 ml of the buffer C described above, 100 µl of a 300 mM-D-glucose solution (final concn. 10 mM) and 100 µl of a 150 mM-sodium acetate solution (final concn. 5 mM), and were pre-incubated and gassed with O_2:CO_2 (95%:5%) for 10 minutes in a shaking water bath at 40°C. ^3H_2O (1 - 2 mCi/flask) was added as a 100 µl aliquot at the time of adding the liver slices to the flask to minimize evaporation of the radioisotope. Initial experiments demonstrated a suitable incubation time of 1 hour. The 'synthetic top phase' used to wash the bottom phases of the lipid extractions did not contain sodium acetate. All other procedures were as presented for the [1-^{14}C] acetate incorporation studies.

3. [9,10-^{3}H] Palmitate incorporation studies

Preparation of [9,10-^{3}H] palmitate-albumin complex

A modified Krebs-Ringer phosphate buffer, pH 7·4, was made up as follows:-

Component	Volume (ml)	Final concn. (mM)
4·5% (w/v) NaCl	20	123·20
5·75% (w/v) KCl	4	24·68
2·11% (w/v) KH_2PO_4	1	1·24
3·82% (w/v) $MgSO_4.7H_2O$	1	1·24
0·1 M-sodium phosphate buffer, pH 7·4	25	20
H_2O	74	
Total volume:-	125	

Bovine serum albumin (fatty acid poor) was dissolved in Krebs-Ringer phosphate buffer, pH 7·4, to a concentration of 120 mg/ml.

Palmitic acid (125 µmoles) was solubilized in chloroform and 125 µCi [9,10-^{3}H] palmitic acid (in toluene) were added. The mixture

was evaporated to dryness under nitrogen. This was followed by the addition of 0·2 M-NaOH (150 µmoles), and the mixture was heated at 60 - 65°C with gentle mixing until the palmitic acid dissolved. The solution was cooled to approximately 45°C, and Krebs-Ringer phosphate buffer containing bovine serum albumin (BSA) was added with mixing. The resulting solution was approximately 5 mM-[9,10-^{3}H] palmitate (5 µCi/ml) in Krebs-Ringer phosphate buffer (pH 7·4) containing BSA (fatty acid poor) at 120 mg/ml.

Incubation procedures

Incubation procedures were as described for the [1-^{14}C] acetate incorporation studies with some modifications. Buffer C was made up as follows, since additional Na^{+} ions were not added to incubations:-

Component	Volume (ml)
4·5% (w/v) NaCl	20
5·75% (w/v) KCl	4
6·1% (w/v) $CaCl_2$	0·6
3·82% (w/v) $MgSO_4.7H_2O$	1
2·11% (w/v) KH_2PO_4	1
1·3% (w/v) $NaHCO_3$	25
H_2O	74·1
Total volume:-	125·7

Incubation flasks contained 2·5 ml of the buffer C described above, 100 µl of a 360 mM-β-D(-)-fructose solution (final concn. 12 mM) and 400 µl of tritiated palmitate-albumin solution, and were pre-incubated and gassed with O_2:CO_2 (95%:5%) for 10 minutes in a shaking water bath at 40°C. Preliminary experiments were carried out to determine a suitable palmitate concentration (0·65 mM) and incubation time (1 hour). Thereafter, incubations were performed in triplicate for each liver.

Reactions were stopped by rapid filtration under suction using a Millipore filter fitted with filter paper discs to enable rapid and efficient removal of liver slices. The slices were immediately washed with 20 ml ice-cold 0·9% (w/v) NaCl, and rapidly placed into a pre-weighed stoppered vial containing 7·5 ml chloroform/methanol (1:2, v/v). This operation lasted approximately 20 seconds. In initial experiments to determine future incubation conditions, the slices from each flask were put into an individual vial. Thereafter, the slices from triplicate incubations were pooled. Vials were re-weighed after the addition of slices to determine the exact weight of tissue. Vials were stored for up to a week at -20°C until lipid extractions were carried out.

Lipid extraction procedures

The vial contents (liver slices (100 - 350 mg, wet wt.) + 7·5 ml chloroform/methanol (1:2, v/v)) were homogenized with 0·16 ml conc. HCl and a variable volume of water (1·65 - 1·9 ml) depending on the weight of slices. Lipid was extracted from the homogenates by the method of Hajra *et al.* (390). Chloroform (2·5 ml) was added to each homogenate in a stoppered extraction tube and the contents were mixed thoroughly for 30 seconds. Then 2 M-KCl in 0·2 M-H_3PO_4 (2·5 ml) was added and the tube contents were mixed for 30 seconds again. Subsequently, the tubes were left on ice until the phases separated. The bottom phase of the extraction was not washed with 'synthetic top phase', but was removed and evaporated to dryness under nitrogen. The dried lipid was dissolved in a small volume of chloroform, from which samples were taken for t.l.c. analysis. Procedures for t.l.c. analysis were as described previously.

4. Statistical analysis

Standard errors are provided to show the degree of variance in the

data. Data were analyzed statistically by Student's 't' test, and levels of statistical significance are indicated where appropriate. Probability values (P) of 0·05 or less were considered to be significant.

RESULTS

1. [1-^{14}C] Acetate incorporation studies

Preliminary experiments were performed to establish suitable conditions for studying the incorporation of [1-^{14}C] acetate into total lipid by liver slices from control and oestrogen-treated male chicks. Oestrogenized birds received a single intramuscular injection of 1 mg 17 β-oestradiol in propane-1,2-diol/100 g body weight approximately 48 hours before death. Control birds received an equivalent volume of propane-1,2-diol only.

Initially, a suitable incubation time was determined using an acetate concentration of 10 mM, since Leveille (239) and Goodridge (276) reported maximum rates of incorporation of acetate into fatty acids by liver slices and hepatocytes, respectively, using this concentration. The effect of incubation time (0 - 90 min) on the incorporation of 10 mM-sodium [1-^{14}C] acetate into total lipid by liver slices from control and oestrogen-treated chicks is shown in Fig. 11. The data show that, after a lag period of about 15 minutes, chick liver slices synthesized lipid from [1-^{14}C] acetate in a linear fashion until at least 60 minutes. Liver slices from control chicks continued to synthesize lipid from [1-^{14}C] acetate in a linear fashion until 90 minutes, but the results for liver slices from oestrogen-treated chicks indicated that linearity was lost with incubation periods of 75 and 90 minutes.

As a result of these observations, an incubation period of 60 minutes

FIGURE 11

The effect of incubation time on the incorporation of 10 mM-[1-^{14}C] acetate into total lipid by liver slices from control and oestrogen-treated chicks

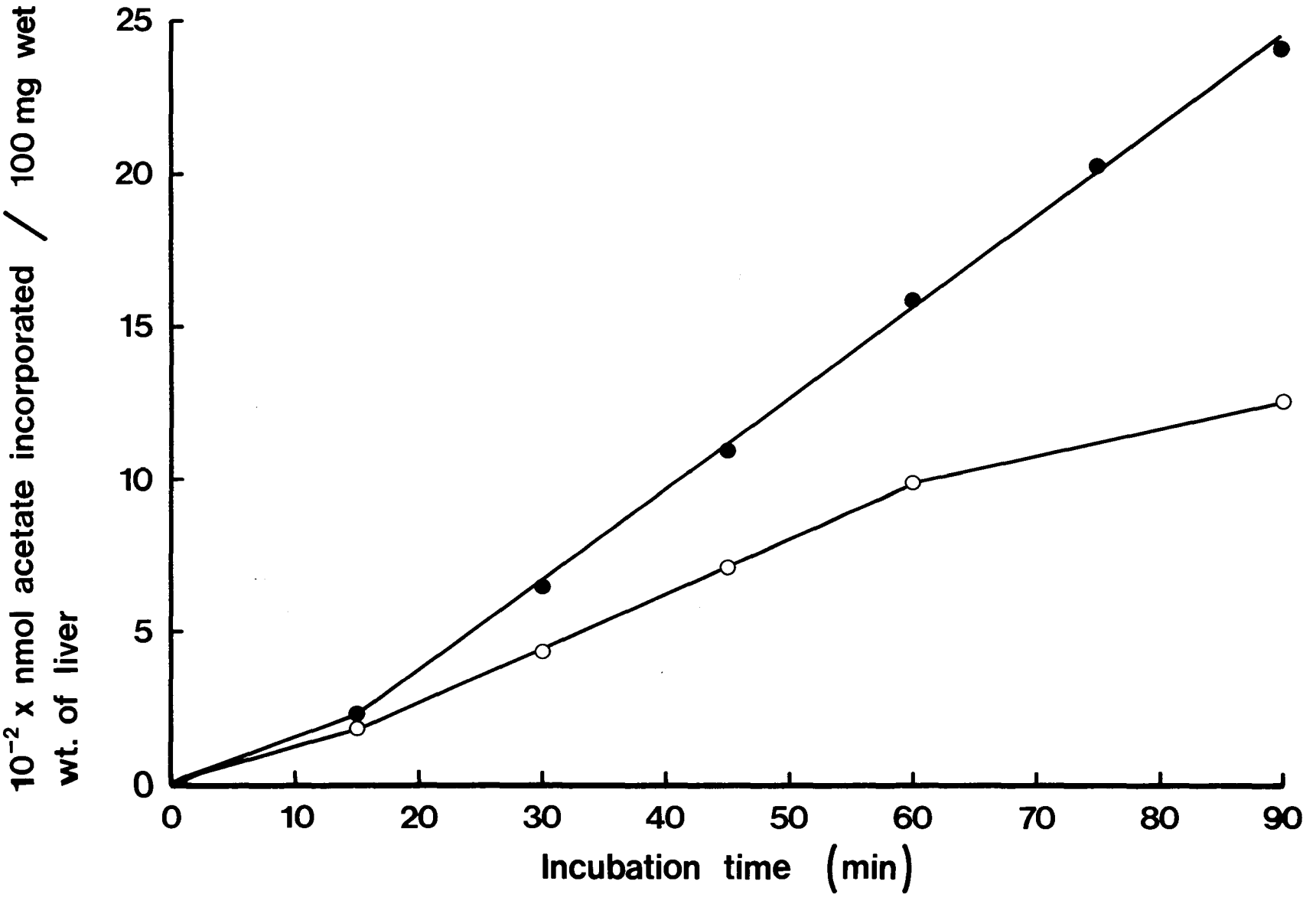
Each oestrogen-treated chick received a single intramuscular injection of 1 mg 17β-oestradiol in propane-1,2-diol/100 g body weight 48 hours before death. Control chicks received an equivalent volume of propane-1,2-diol only 48 hours before death.

Chick liver slices (150 ± 10 mg, wet wt.) were incubated at 40°C in 3 ml avian bicarbonate buffer, pH 7·4, containing 10 mM-sodium [1-^{14}C] acetate (1·5 μCi). Total lipid was extracted at the times indicated.

● values for control chicks

○ values for oestrogen-treated chicks

Each value represents the average of single determinations for 4 birds.


10^{-2} x nmol acetate incorporated / 100 mg wet wt. of liver
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
90
Incubation time (min)

was adopted during the study of the effect of acetate concentration on the incorporation of [1-^{14}C] acetate into total lipid by chick liver slices. Liver slices were incubated in the presence of [1-^{14}C] acetate concentrations ranging from 1 to 20 mM, and maximum incorporation was obtained with an acetate concentration of 10 mM (Fig. 12). All subsequent incubations involving the incorporation of [1-^{14}C] acetate into total lipid by liver slices from control and oestrogen-treated chicks were performed for 1 hour in the presence of 10 mM-sodium [1-^{14}C] acetate.

Experiments were performed to investigate the incorporation of 10 mM-[1-^{14}C] acetate into total lipid by liver slices from male chicks treated with a single intramuscular injection of 1 mg 17β-oestradiol in propane-1,2-diol/100 g body weight, or with an equivalent volume of propane-1,2-diol only. Birds were sacrificed at varying times after injection, and liver slice incubations were conducted as described in the Methods section. Chicks were also included that had received multiple injections over several days, and which were sacrificed 24 hours after the last injection. Incubations were performed in triplicate for each liver. The results of these experiments are presented in Figs. 13 - 15 and in Tables 6 and 7. In Tables 6 and 7, the chicks have been allocated to groups dependent upon the time of death ($3\frac{3}{4}$ - $73\frac{1}{2}$ h) after a single injection, or upon the number of injections received (3 - 11). Wherever possible, the results for each group of oestrogen-treated chicks have been compared with the results for the corresponding group of control chicks. The data were analyzed statistically by Student's 't' test, and levels of statistical significance are indicated in the tables and text where appropriate.

In Fig. 13 and Tables 6 and 7, the results of the experiments have been expressed as nmoles acetate incorporated/100 mg liver/hour. The

FIGURE 12

The effect of acetate concentration on the incorporation of [1-^{14}C] acetate into total lipid by chick liver slices

Each oestrogen-treated chick received a single intramuscular injection of 1 mg 17β-oestradiol in propane-1,2-diol/100 g body weight 48 hours before death. Control chicks received an equivalent volume of propane-1,2-diol only 48 hours before death.

Chick liver slices (150 ± 10 mg, wet wt.) were incubated in 3 ml avian bicarbonate buffer, pH 7·4, containing varying concentrations (1 - 20 mM) of sodium [1-^{14}C] acetate (1·5 μCi/incubation). Total lipid was extracted after a 60 minute incubation at 40°C.

Each value represents the average of single determinations for 4 control chicks and 3 oestrogen-treated chicks.

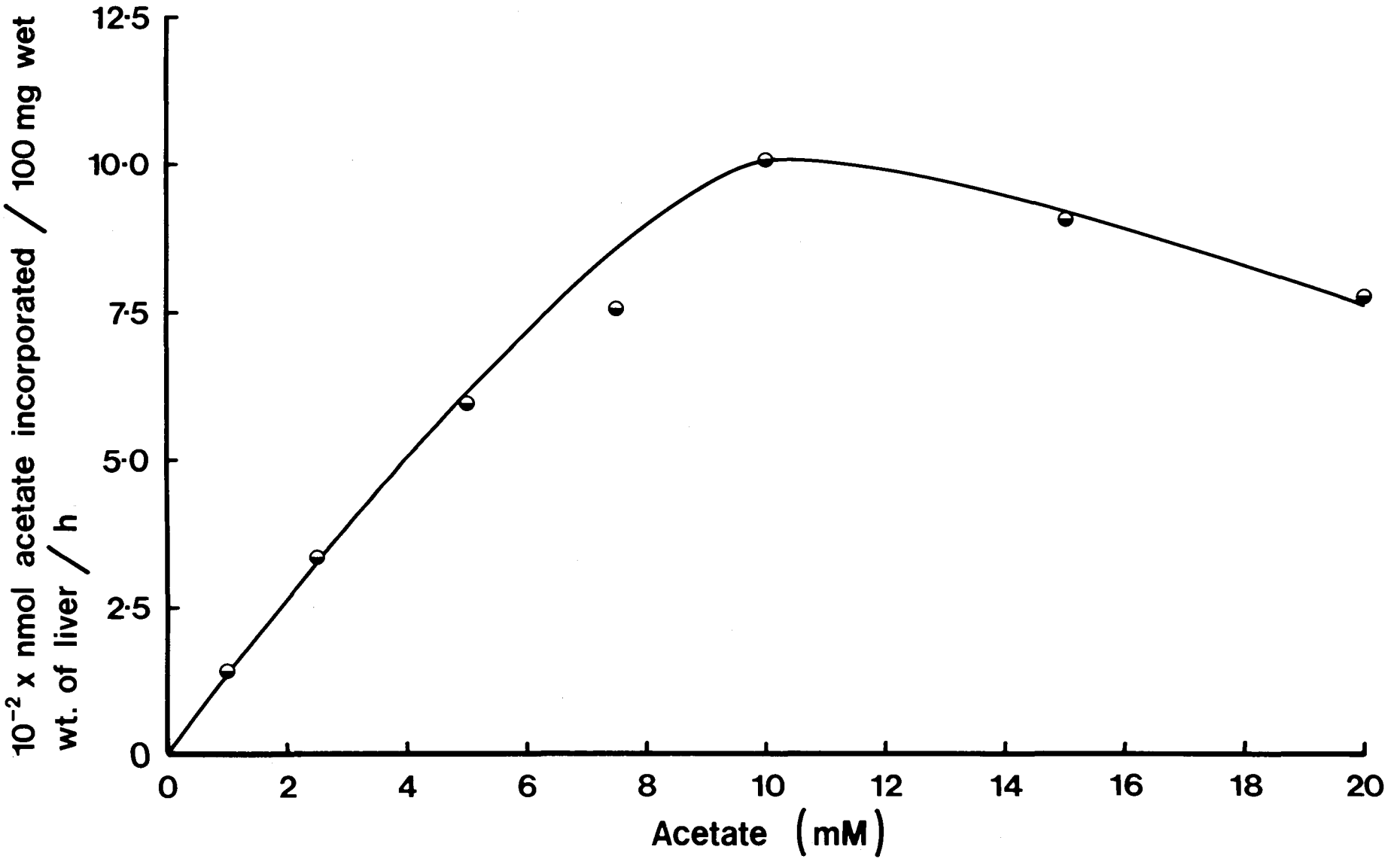

12·5
10·0
7·5
5·0
2·5
0
10^{-2} x nmol acetate incorporated / 100 mg wet wt. of liver / h
0
2
4
6
8
10
12
14
16
18
20
Acetate (mM)

FIGURE 13

The incorporation of 10 mM-[1-^{14}C] acetate into total lipid by liver slices (nmol acetate incorporated/100 mg liver/h) from control and oestrogen-treated male chicks at varying times after injection

Oestrogen-treated chicks received a single intramuscular injection of 1 mg 17β-oestradiol in propane-1,2-diol/100 g body weight. Control chicks received an equivalent volume of propane-1,2-diol only. At the indicated times after injection, birds were sacrificed and liver slices were prepared. Chicks receiving multiple injections were injected every day for variable periods of time (3 - 11 days), and were sacrificed 24 hours after the last injection. The chicks that received 4 injections were an exception, since these birds received injections on alternate days over 7 days, and were killed 24 hours after the last injection.

Liver slices (150 ± 10 mg, wet wt.) were incubated in 3 ml avian bicarbonate buffer, pH 7·4, containing 10 mM-sodium [1-^{14}C] acetate (1·5 - 3 μCi/incubation). Total lipid was extracted after a 60 minute incubation at 40°C.

● values for control chicks

○ values for oestrogen-treated chicks

Each value represents the average of 3 determinations for a single bird.

Chicks were aged 2 - 5 weeks.

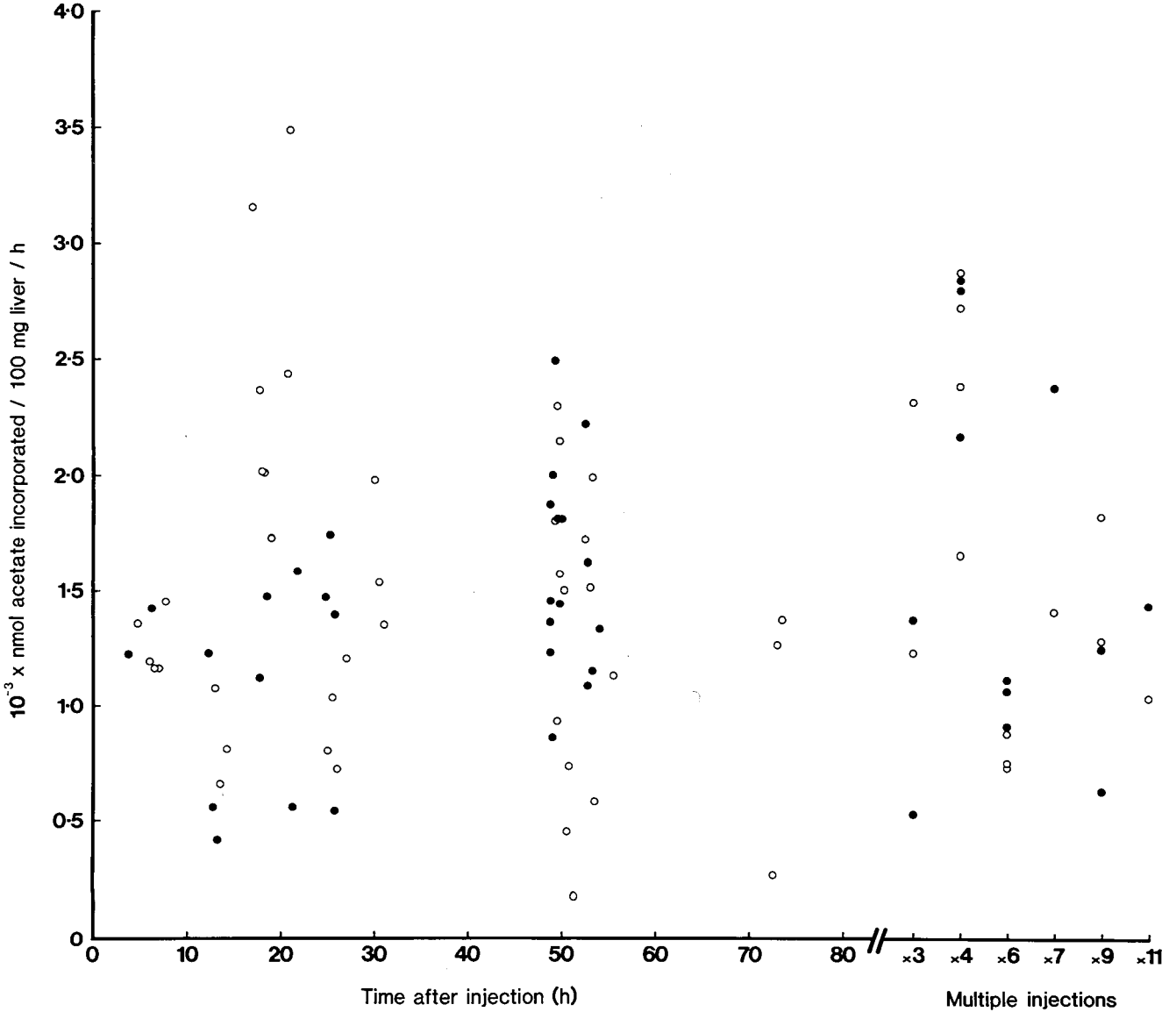

4·0
3·5
3·0
2·5
2·0
1·5
1·0
0·5
0
10^{-3} x nmol acetate incorporated / 100 mg liver / h
0
10
20
30
40
50
60
70
80
×3
×4
×6
×7
×9
×11
Time after injection (h)
Multiple injections

FIGURE 14

The incorporation of 10 mM-[1-^{14}C] acetate into total lipid by liver slices (nmol acetate incorporated/liver/min) from control and oestrogen-treated male chicks at varying times after injection

See legend of Fig. 13 for experimental details.

● values for control chicks

○ values for oestrogen-treated chicks

Each value represents the average of 3 determinations for a single bird.

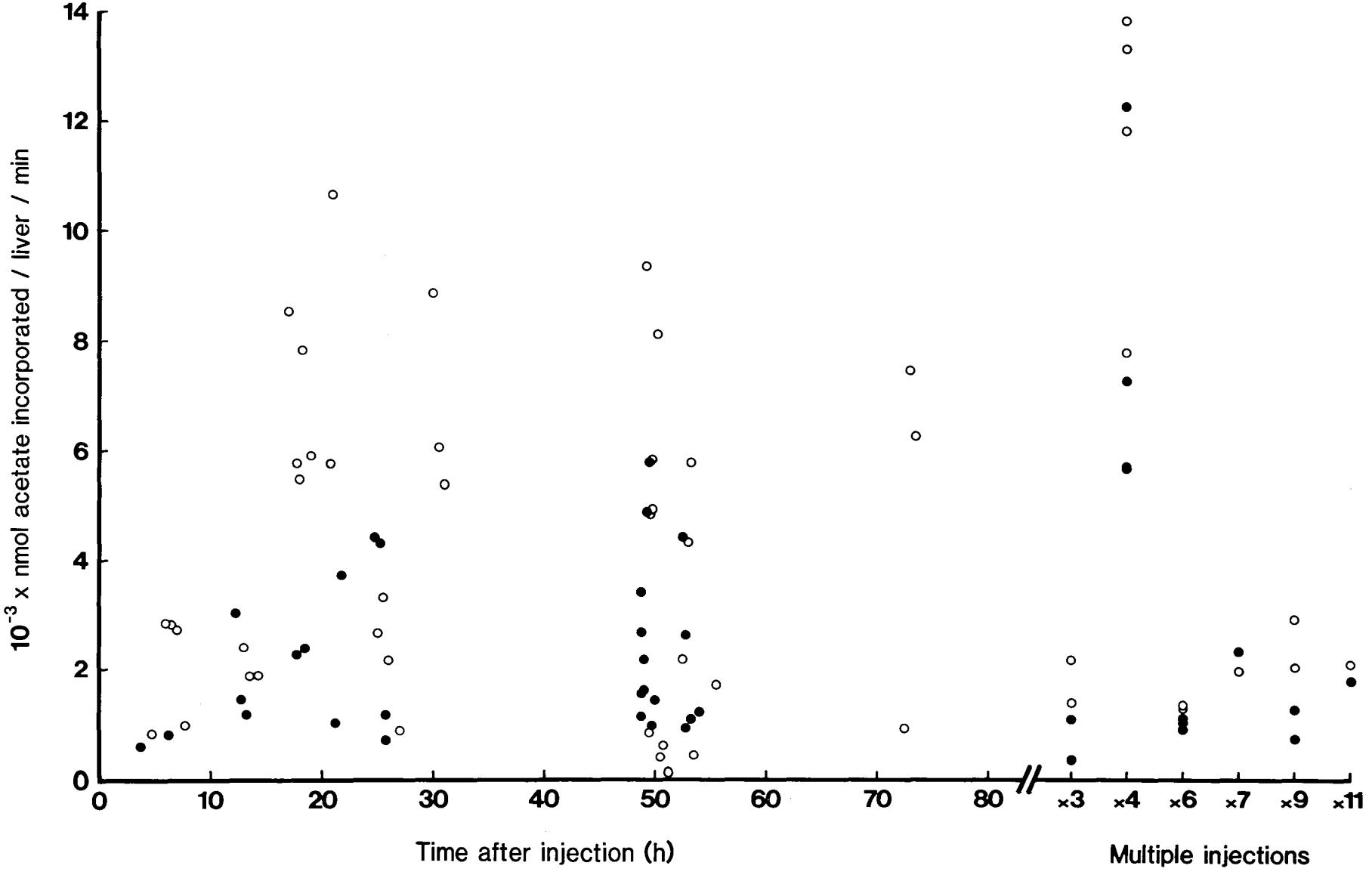

14
12
10
8
6
4
2
0
10^{-3} x nmol acetate incorporated / liver / min
0
10
20
30
40
50
60
70
80
×3
×4
×6
×7
×9
×11
Time after injection (h)
Multiple injections

FIGURE 15

The incorporation of 10 mM-[1-^{14}C] acetate into total lipid by liver slices (nmol acetate incorporated/0·1 mg liver DNA/h) from control and oestrogen-treated male chicks at varying times after injection

See legend of Fig. 13 for experimental details.

● values for control chicks

○ values for oestrogen-treated chicks

Each value represents the average of 3 determinations for a single bird.

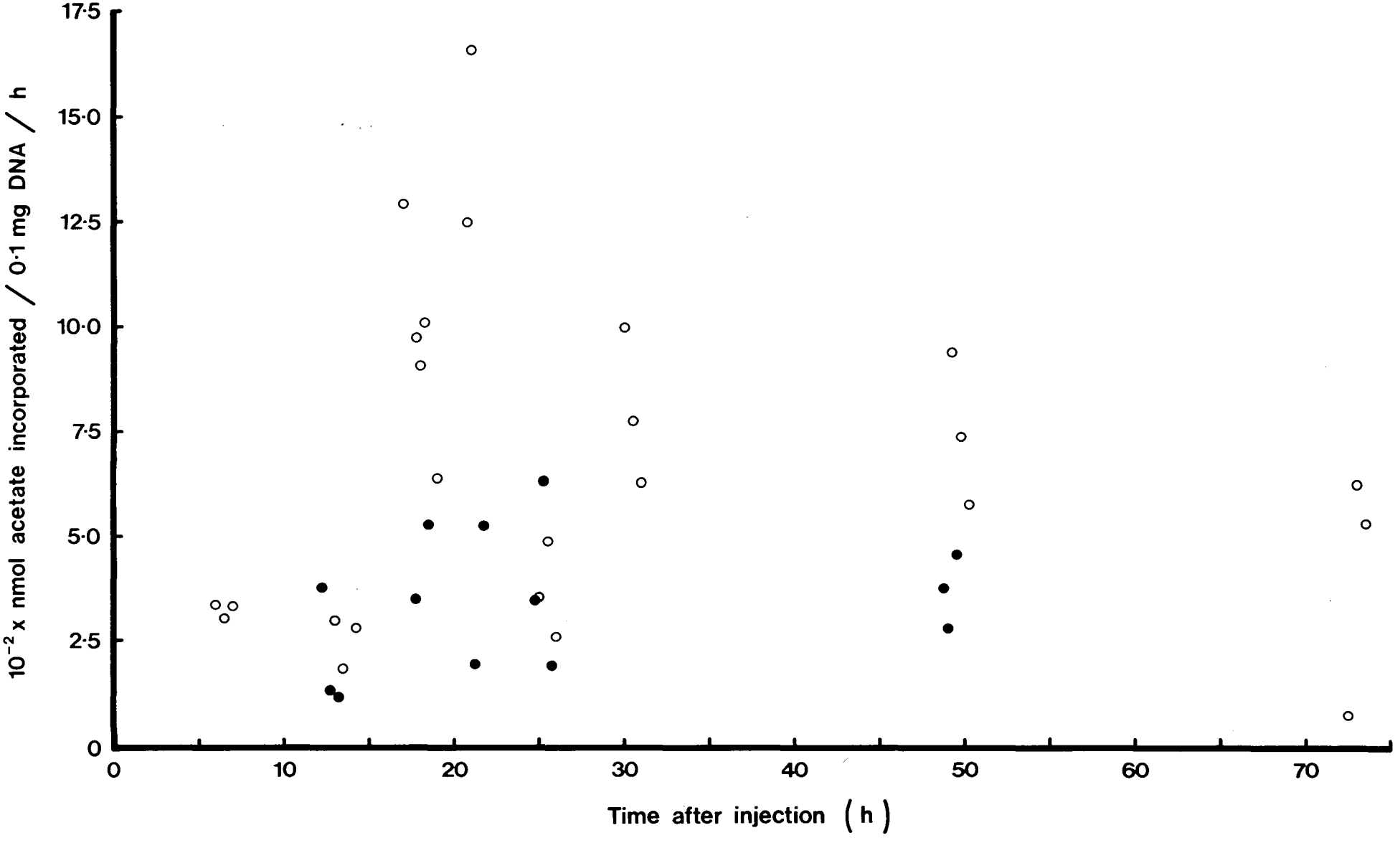

17·5
15·0
12·5
10·0
7·5
5·0
2·5
0
10^{-2} x nmol acetate incorporated / 0·1 mg DNA / h
0
10
20
30
40
50
60
70
Time after injection (h)

TABLE 6

The incorporation of 10 mM-[1-^{14}C] acetate into total lipid by liver slices from male chicks at various times after a single injection of 17 β-oestradiol (1 mg/100 g body wt.) in propane-1,2-diol, or of an equivalent volume of propane-1,2-diol only

Incorporation of [1-^{14}C] acetate		Time after injection (h)						
		3¾ - 7¾	12¼ - 14¼	17 - 21¾	24¾ - 27	30 - 31	48¾ - 55½	72½ - 73½
nmol acetate/ 100 mg liver/h	E	1266 ± 60 (5)	849 ± 122 (3)	2458 ± 243 (7) ††	944 ± 111 (4)	1623 ± 186 (3)	1327 ± 178 (14)	969 ± 352 (3)
	C	1324 ± 99 (2)	734 ± 251 (3)	1186 ± 231 (4)	1288 ± 260 (4)	N.D.	1586 ± 116 (15)	N.D.
nmol acetate/ liver/min	E	2035 ± 470 (5)	2047 ± 177 (3)	7133 ± 740 (7) Δ	2259 ± 519 (4)	6772 ± 1070 (3)	3533 ± 820 (14)	4876 ± 2012 (3)
	C	688 ± 106 (2)	1881 ± 585 (3)	2348 ± 555 (4)	2647 ± 997 (4)	N.D.	2393 ± 404 (15)	N.D.
nmol acetate/ 0·1 mg DNA/h	E	323 ± 10 (3)	254 ± 36 (3)	1103 ± 124 (7) ††	367 ± 66 (3)	799 ± 108 (3)	750 ± 105 (3) †	409 ± 170 (3)
	C	N.D.	208 ± 85 (3)	399 ± 80 (4)	390 ± 129 (3)	N.D.	372 ± 51 (3)	N.D.
Liver wt. (as % of body wt.)	E	4·06 ± 0·35 (5)	3·80 ± 0·29 (3)	4·60 ± 0·16 (7)	4·69 ± 0·16 (4)	5·47 ± 0·07 (3)	5·04 ± 0·22 (14)	5·38 ± 0·57 (3)
	C	4·47 ± 0·40 (2)	3·70 ± 0·02 (3)	3·45 ± 0·16 (4)	3·69 ± 0·29 (4)	N.D.	3·78 ± 0·13 (15)	N.D.

See legend of Fig. 13 for experimental details.

Incubations were performed in triplicate, and the results are the means (± S.E.M.) of the average values obtained from 2 - 15 chicks. The number of birds involved in each group is given in parentheses.

E = values for oestrogen-treated chicks

C = values for control chicks

N.D. = not determined

Data were analyzed statistically by Student's 't' test, and levels of statistical significance are presented with respect to values for corresponding control chicks.

† significant at $P < 0{\cdot}05$

†† significant at $P < 0{\cdot}01$

Δ significant at $P < 0{\cdot}002$

TABLE 7

The effect of multiple injections on the incorporation of 10 mM-[1-^{14}C] acetate into total lipid by liver slices from control and oestrogen-treated male chicks

Incorporation of [1-^{14}C] acetate		number of injections					
		3	4	6	7	9	11
nmol acetate/ 100 mg liver/h	E	1773 ± 542 (2)	2408 ± 272 (4)	790 ± 46 (3) †	1409 (1)	1551 ± 269 (2)	1036 (1)
	C	953 ± 422 (2)	2896 ± 332 (4)	1031 ± 61 (3)	2376 (1)	941 ± 308 (2)	1434 (1)
nmol acetate/ liver/min	E	1772 ± 396 (2)	11683 ± 1370 (4)	1298 ± 21 (3) ††	1951 (1)	2468 ± 438 (2)	2085 (1)
	C	715 ± 366 (2)	7718 ± 1560 (4)	1007 ± 59 (3)	2325 (1)	996 ± 259 (2)	1793 (1)
Liver wt. (as % of body wt.)	E	5·70 ± 0·18 (2)	7·02 ± 0·31 (4)	4·66 ± 0·25 (3)	6·14 (1)	5·71 ± 0·42 (2)	5·86 (1)
	C	4·24 ± 0·15 (2)	3·71 ± 0·18 (4)	3·03 ± 0·19 (3)	4·26 (1)	3·62 ± 0·08 (2)	3·95 (1)

See legend of Fig. 13 for experimental details.

Incubations were performed in triplicate, and the results are the average values for a single bird or the means (± S.E.M.) of the average values obtained from 2 - 4 birds. The number of birds involved in each group is given in parentheses.

E = values for oestrogen-treated chicks

C = values for control chicks

Data were analyzed statistically by Student's 't' test, and levels of statistical significance are presented with respect to values for corresponding control birds.

† significant at $P < 0{\cdot}05$

†† significant at $P < 0{\cdot}01$

data illustrated in Fig. 13 clearly show the enormous variability among birds, which makes any differences between control and oestrogen-treated chicks difficult to detect. The values obtained at 17 - 21¾ hours after a single injection of 1 mg 17 β-oestradiol/100 g body weight were significantly greater ($P < 0{\cdot}01$) than the values for the corresponding control group (Table 6). The mean value for this group of oestrogen-treated chicks was more than double that for the corresponding group of control birds. Since there were no control groups to compare directly with the 30 - 31 h and 72½ - 73½ h oestrogen-treated groups, comparisons were made with all the other control groups. The results for the 30 - 31 h oestrogen-treated group were significantly greater ($P < 0{\cdot}05$) than the results for the 12¼ - 14¼ h control group, but did not attain significance with respect to the other control groups. The results for the 72½ - 73½ h oestrogen-treated group failed to attain significance with respect to any of the control groups, and the values for the remaining oestrogen-treated groups were not significantly different from those of their corresponding control groups. The results obtained for chicks treated with a daily injection of 1 mg 17 β-oestradiol/100 g body weight for 6 days, and sacrificed on the 7th day, were significantly lower ($P < 0{\cdot}05$) than the results for the corresponding group of control birds (Table 7). However, the results for the other multiply-injected groups of oestrogen-treated birds were not significantly different from the results for the corresponding control chicks.

In Fig. 14 and Tables 6 and 7, the results of the experiments have been expressed as nmoles acetate incorporated/liver/minute. Again, quite a lot of variability was evident, but from about 17 hours after a single oestrogen injection the acetate incorporation values had a tendency to be greater than control values (Fig. 14). The values obtained at 17 - 21¾ hours after a single injection of 17 β-oestradiol

TABLE 8

The incorporation of [1-^{14}C] acetate and $^{3}H_2O$ into the fatty acids of complex lipids and into non-saponifiable lipids by liver slices from control and oestrogen-treated male chicks

Radioisotope and labelled compound	Treatment of chicks	Percentage recovery of radioactivity in the procedure	Percentage of the recovered radioactivity in non-saponifiable lipids	Percentage of the recovered radioactivity in fatty acids derived from complex lipids
[1-^{14}C] acetate	Control	90·47 ± 2·64 (10)	9·92 ± 0·98 (10)	90·08 ± 0·98 (10)
	Oestrogenized	91·57 ± 0·79 (15)	15·91 ± 2·00 (15) †	84·09 ± 2·00 (15) †
$^{3}H_2O$	Control	80·10 ± 1·24 (3)	4·18 ± 0·62 (3)	95·82 ± 0·62 (3)
	Oestrogenized	78·13 ± 1·12 (3)	6·53 ± 1·78 (3)	93·47 ± 1·78 (3)

Values are the means (± S.E.M.) of the results for 3 - 15 chicks.

The number of observations involved in each group is given in parentheses.

Oestrogen-treated chicks received a single intramuscular injection of 1 mg 17β-oestradiol in propane-1,2-diol/100 g body weight. Control chicks received an equivalent volume of propane-1,2-diol only.

At various times after injection, chicks were sacrificed and liver slices were prepared. Liver slices (150 ± 10 mg, wet wt.) were incubated in 3 ml avian bicarbonate buffer, pH 7·4, containing 10 mM-sodium [1-^{14}C] acetate (1·5 - 3 μCi/incubation), or in 3 ml avian bicarbonate buffer, pH 7·4, containing 10 mM-D-glucose, 5 mM-sodium acetate and $^{3}H_2O$ (1 - 2 mCi/incubation). Total lipid was extracted after a 60 minute incubation at 40°C. The saponification procedure and extractions were performed as described in the Methods section.

Data were analyzed statistically by Student's 't' test, and levels of statistical significance are presented for results from oestrogen-treated birds with respect to values for corresponding control birds.

† significant at $P < 0{\cdot}05$

TABLE 9

The incorporation of [1-^{14}C] acetate and $^{3}H_2O$ into lipid classes, as separated by thin layer chromatography, by liver slices from control and oestrogen-treated male chicks

Radioisotope and labelled compound	Treatment of chicks	Percentage recovery of radioactivity on t.l.c. plates	Percentage of recovered radioactivity in:-				
			Phospholipid	Monoacyl-glycerol	Diacylglycerol + cholesterol	Free fatty acid	Triacylglycerol
[1-^{14}C] acetate	Control	104·19 ± 4·60 (8)	16·41 ± 1·19 (8)	1·70 ± 0·70 (8)	8·64 ± 1·06 (8)	0·48 ± 0·15 (8)	66·46 ± 3·73 (8)
	Oestrogenized	106·50 ± 2·34 (12)	13·02 ± 0·72‡ (12)	1·83 ± 0·44 (12)	7·52 ± 1·05 (12)	0·90 ± 0·27 (12)	69·50 ± 2·01 (12)
$^{3}H_2O$	Control	65·41 ± 1·90 (3)	6·09 ± 0·47 (3)	0·19 ± 0·04 (3)	4·36 ± 0·61 (3)	0·38 ± 0·04 (3)	86·91 ± 0·93 (3)
	Oestrogenized	64·50 ± 1·35 (3)	6·04 ± 0·39 (3)	0·18 ± 0·02 (2)	4·11 ± 0·23 (3)	0·44 ± 0·03 (3)	87·19 ± 0·75 (3)

Values are the means (± S.E.M.) of the results for 2 - 12 chicks.

The number of observations involved in each group is given in parentheses.

See legend of Table 8 for experimental details.

Liver slice incubations, lipid extractions and t.l.c. analyses were performed as described in the Methods section.

Data were analyzed statistically by Student's 't' test, and levels of statistical significance are presented for results from oestrogen-treated birds with respect to values for corresponding control birds.

‡ significant at $P < 0{\cdot}02$

the label was located in triacylglycerol, with about 16·4% being located at the origin (phospholipid), 1·7% in monoacylglycerol, 8·6% in diacylglycerol and cholesterol, and about 0·5% in free fatty acids. A similar distribution of the label in the lipid classes was observed for lipid extracts from liver slices of oestrogenized chicks, with about 69·5% of the label being located in triacylglycerol, 13% at the origin (phospholipid), 1·8% in monoacylglycerol, 7·5% in diacylglycerol and cholesterol, and about 0·9% in free fatty acids. A significantly greater percentage of the recovered radioactivity was located in phospholipid after t.l.c. analysis of lipid extracts from control chick liver slices than was obtained for lipid extracts from oestrogen-treated chick liver slices ($P < 0·02$). There were no significant differences between control and oestrogen-treated liver lipid extracts in the percentages of the recovered radioactivity located in the other lipid classes.

2. $^{3}H_2O$ incorporation studies

De novo lipogenesis by liver slices from untreated, control and oestrogen-treated male chicks was also assayed by measuring the incorporation of tritium from $^{3}H_2O$ into total lipid. An advantage of using $^{3}H_2O$ rather than [1-^{14}C] acetate is that hydrogen from water is incorporated into lipids regardless of the nature of the carbon substrate, and consequently the incorporation of tritium from $^{3}H_2O$ provides a measure of total de novo lipogenesis, whereas the use of [1-^{14}C] acetate measures lipogenesis from that substrate alone. A further advantage of using $^{3}H_2O$ is that substrate pool size problems are minimized. Such problems may arise with the use of [1-^{14}C] acetate, and possible differences in pool size were not considered in the present study, since the cellular pool size of acetyl-CoA was not determined.

Initial experiments were performed to establish a suitable incubation period for studying the incorporation of tritium from ^3H_2O into total lipid by liver slices from control and oestrogen-treated male chicks. The effect of incubation time (0 - 60 min) on the incorporation of tritium from ^3H_2O into total lipid by chick liver slices is shown in Fig. 16. The data show that liver slices from control and oestrogen-treated chicks exhibit a lag period of about 10 minutes, after which incorporation of tritium from ^3H_2O into lipid is linear until at least 60 minutes. An incubation period of 60 minutes was, therefore, adopted for all subsequent incubations involving the incorporation of tritium from ^3H_2O into total lipid by chick liver slices.

Experiments were carried out to investigate the incorporation of tritium from ^3H_2O into total lipid by liver slices from untreated chicks, chicks treated with a single intramuscular injection of 1 mg 17β-oestradiol in propane-1,2-diol/100 g body weight, and chicks treated with an equivalent volume of propane-1,2-diol only. Birds were sacrificed at varying times after injection and liver slice incubations were performed as described in the Methods section. Chicks were also included that had received an injection every day for 7 or 11 days and which were sacrificed 24 hours after the last injection. Incubations were performed in triplicate for each liver. The results of these experiments are presented in Figs. 17 - 19 and in Table 10. In Table 10, the chicks have been allocated to groups dependent upon treatment and upon the time of death after a single injection ($3\frac{1}{2}$ - $43\frac{1}{4}$ h), or upon the number of injections received (7 or 11). Wherever possible, the results for each group of oestrogen-treated chicks have been compared with the results for the corresponding group of control chicks. The data were analyzed statistically by Student's 't' test, and levels of statistical significance are indicated where appropriate.

FIGURE 16

The effect of incubation time on the incorporation of tritium from ^3H_2O into total lipid by liver slices from control and oestrogen-treated chicks

Oestrogen-treated chicks received a single intramuscular injection of 17 β-oestradiol in propane-1,2-diol (1 mg hormone/100 g body wt.) 24 hours before death, or daily for 7 days prior to sacrifice. Control chicks received an equivalent volume of propane-1,2-diol daily for 7 days or 11 days prior to sacrifice.

Chick liver slices (150 ± 10 mg, wet wt.) were incubated at 40°C in 3 ml avian bicarbonate buffer, pH 7·4, containing 5 mM-sodium acetate, 10 mM-D-glucose and 2 mCi ^3H_2O. Total lipid was extracted at the times indicated.

(a) values for control chicks

(b) values for oestrogen-treated chicks

Each value represents the average of single or duplicate determinations for 2 chicks.

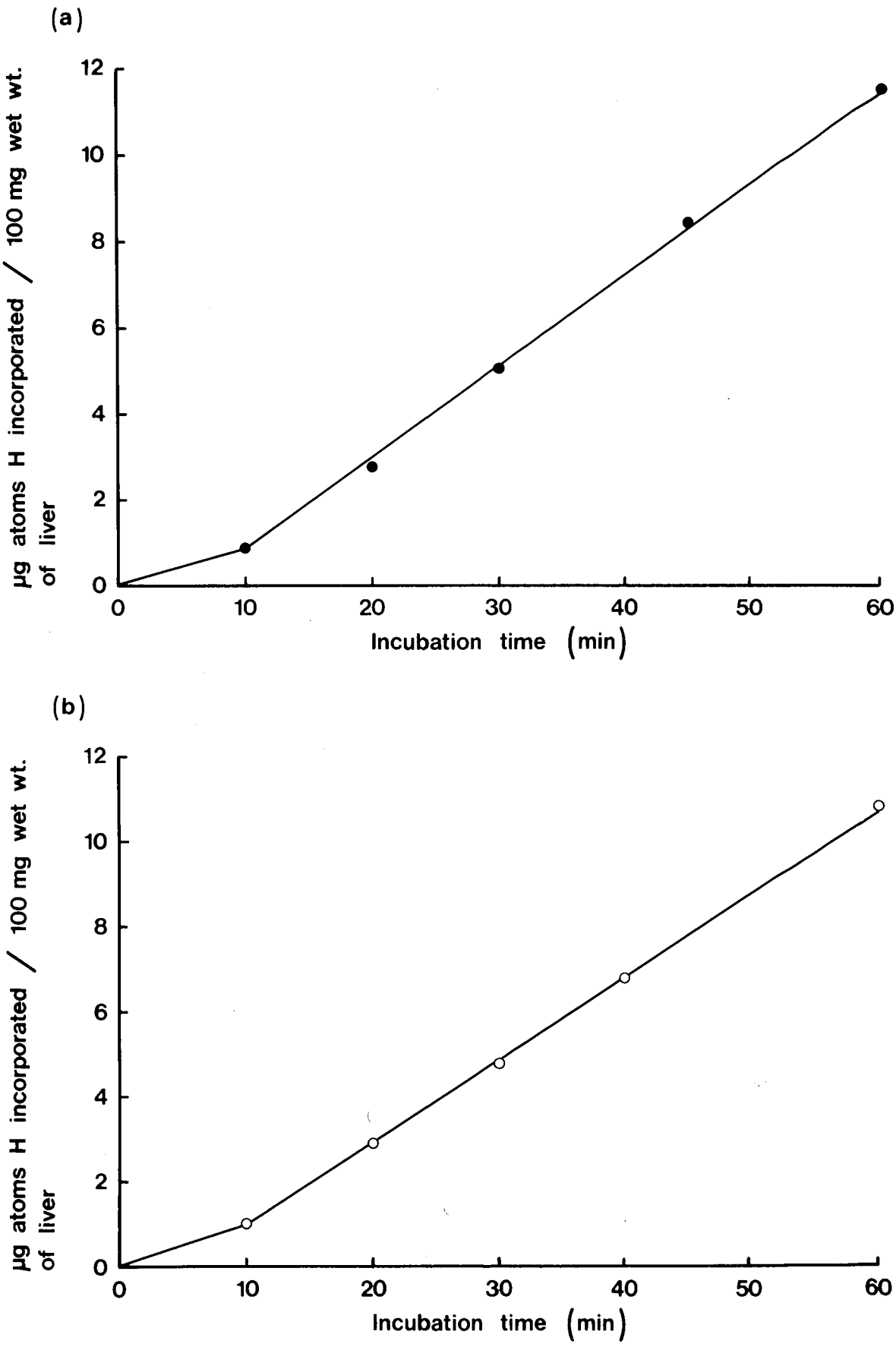

(a)
µg atoms H incorporated / 100 mg wet wt. of liver
0
2
4
6
8
10
12
0
10
20
30
40
50
60
Incubation time (min)
(b)
µg atoms H incorporated / 100 mg wet wt. of liver
0
2
4
6
8
10
12
0
10
20
30
40
50
60
Incubation time (min)

FIGURE 17

The incorporation of tritium from $^{3}H_2O$ into total lipid by liver slices (µg atoms H incorporated/100 mg liver/h) from control and oestrogen-treated male chicks at varying times after injection

Oestrogen-treated chicks received a single intramuscular injection of 1 mg 17 β-oestradiol in propane-1,2-diol/100 g body weight. Control chicks received an equivalent volume of propane-1,2-diol only. Untreated chicks were also included in the experiments. At the indicated times after injection, birds were sacrificed and liver slices were prepared. Chicks receiving multiple injections were injected every day for 7 or 11 days, and were sacrificed 24 hours after the last injection.

Liver slices (150 ± 10 mg, wet wt.) were incubated at 40°C in 3 ml avian bicarbonate buffer, pH 7·4, containing 5 mM-sodium acetate, 10 mM-D-glucose and 1 - 2 mCi $^{3}H_2O$. Total lipid was extracted after a 60 minute incubation.

▲ values for untreated chicks

● values for control chicks

○ values for oestrogen-treated chicks

Each value represents the average of 3 determinations for a single bird.

Chicks were aged 2 - 5 weeks.

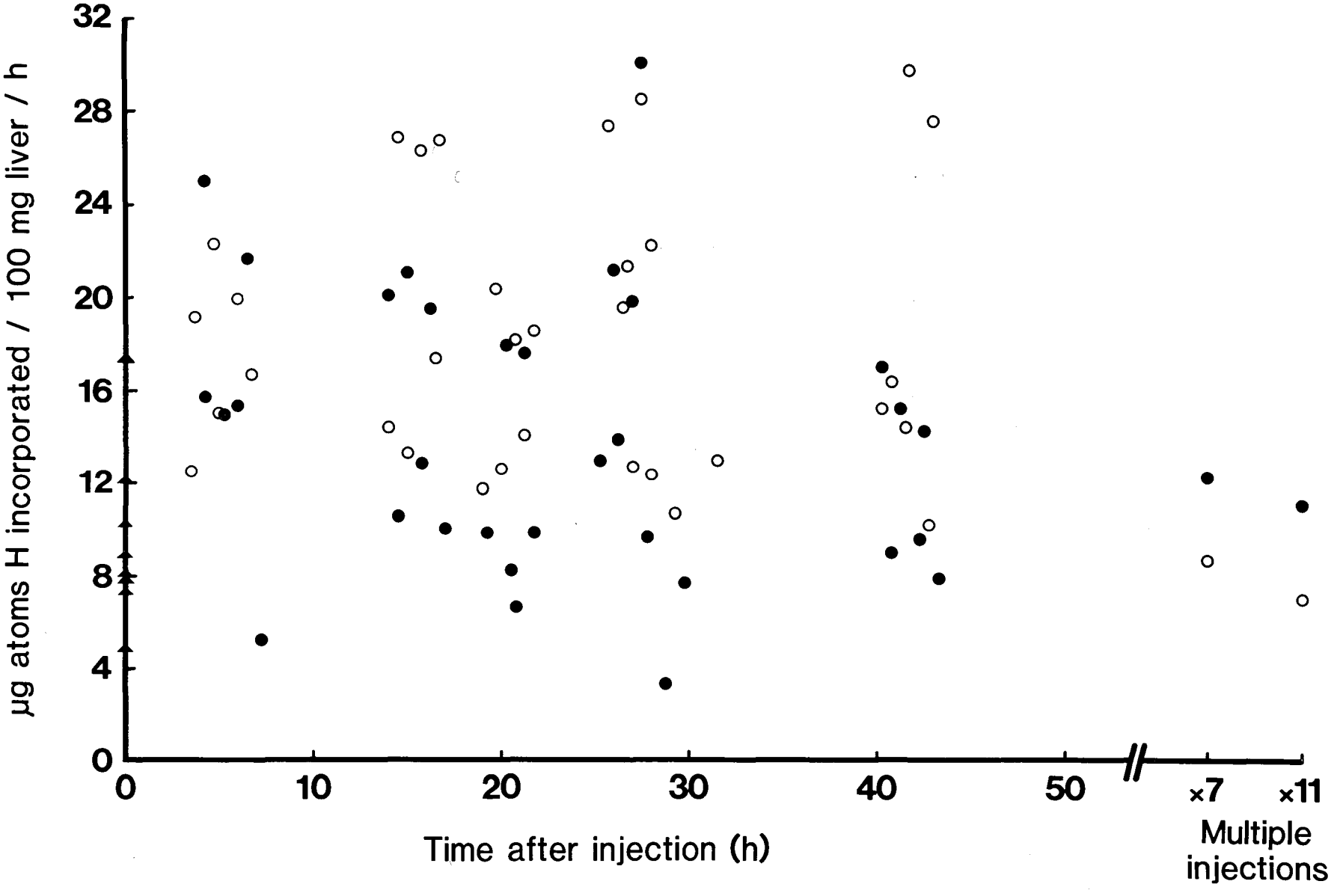

µg atoms H incorporated / 100 mg liver / h
32
28
24
20
16
12
8
4
0
0
10
20
30
40
50
×7
×11
Multiple injections
Time after injection (h)

FIGURE 18

The incorporation of tritium from $^{3}H_2O$ into total lipid by liver slices (μg atoms H incorporated/liver/min) from control and oestrogen-treated male chicks at varying times after injection

See legend of Fig. 17 for experimental details.

▲ values for untreated chicks

● values for control chicks

○ values for oestrogen-treated chicks

Each value represents the average of 3 determinations for a single bird.

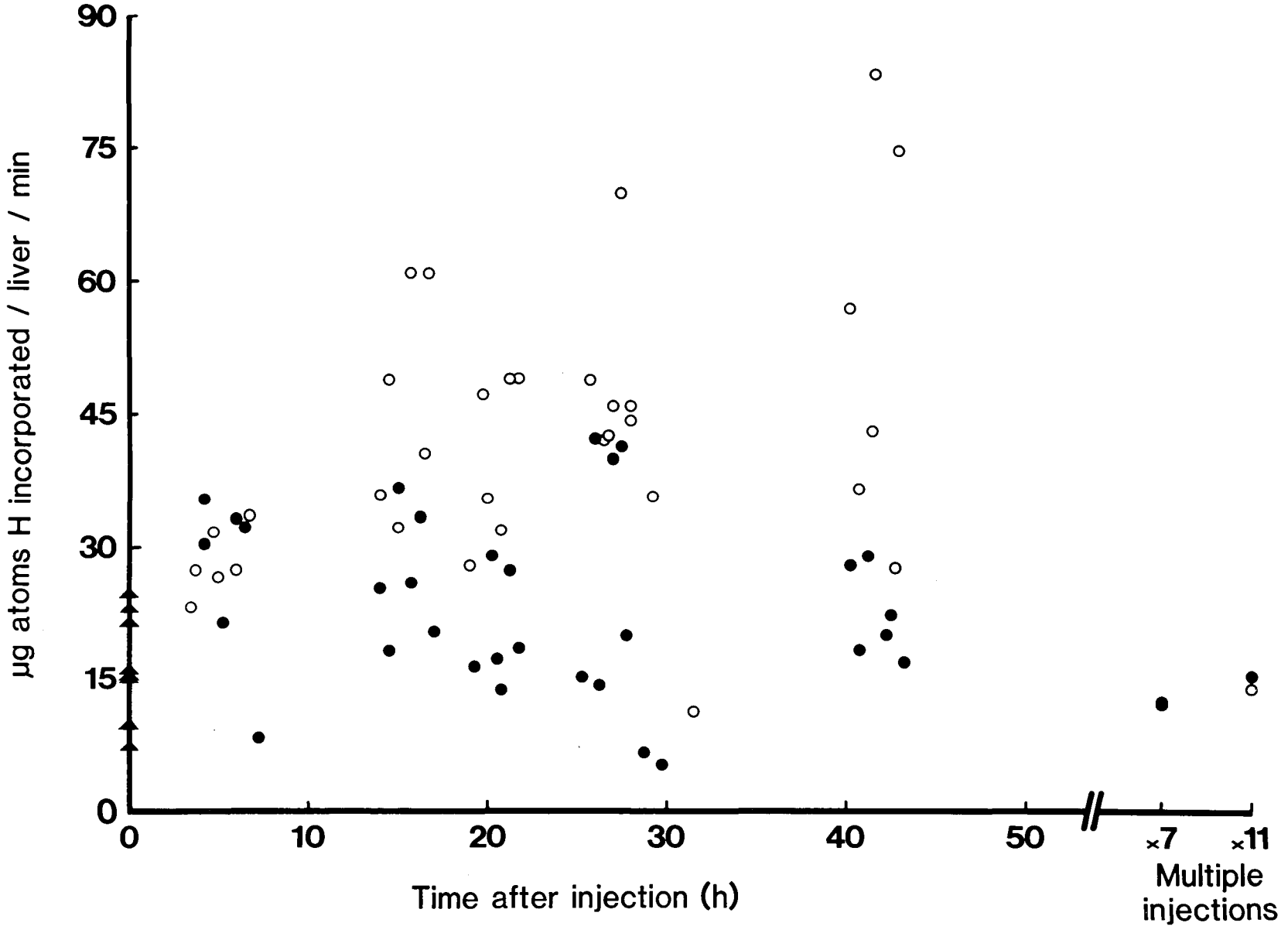

90
75
60
45
30
15
0
µg atoms H incorporated / liver / min
0
10
20
30
40
50
×7
×11
Multiple injections
Time after injection (h)

FIGURE 19

The incorporation of tritium from $^{3}H_2O$ into total lipid by liver slices (μg atoms H incorporated/0·1 mg liver DNA/h) from control and oestrogen-treated male chicks at varying times after injection

See legend of Fig. 17 for experimental details.

▲ values for untreated chicks

● values for control chicks

○ values for oestrogen-treated chicks

Each value represents the average of 3 determinations for a single bird.

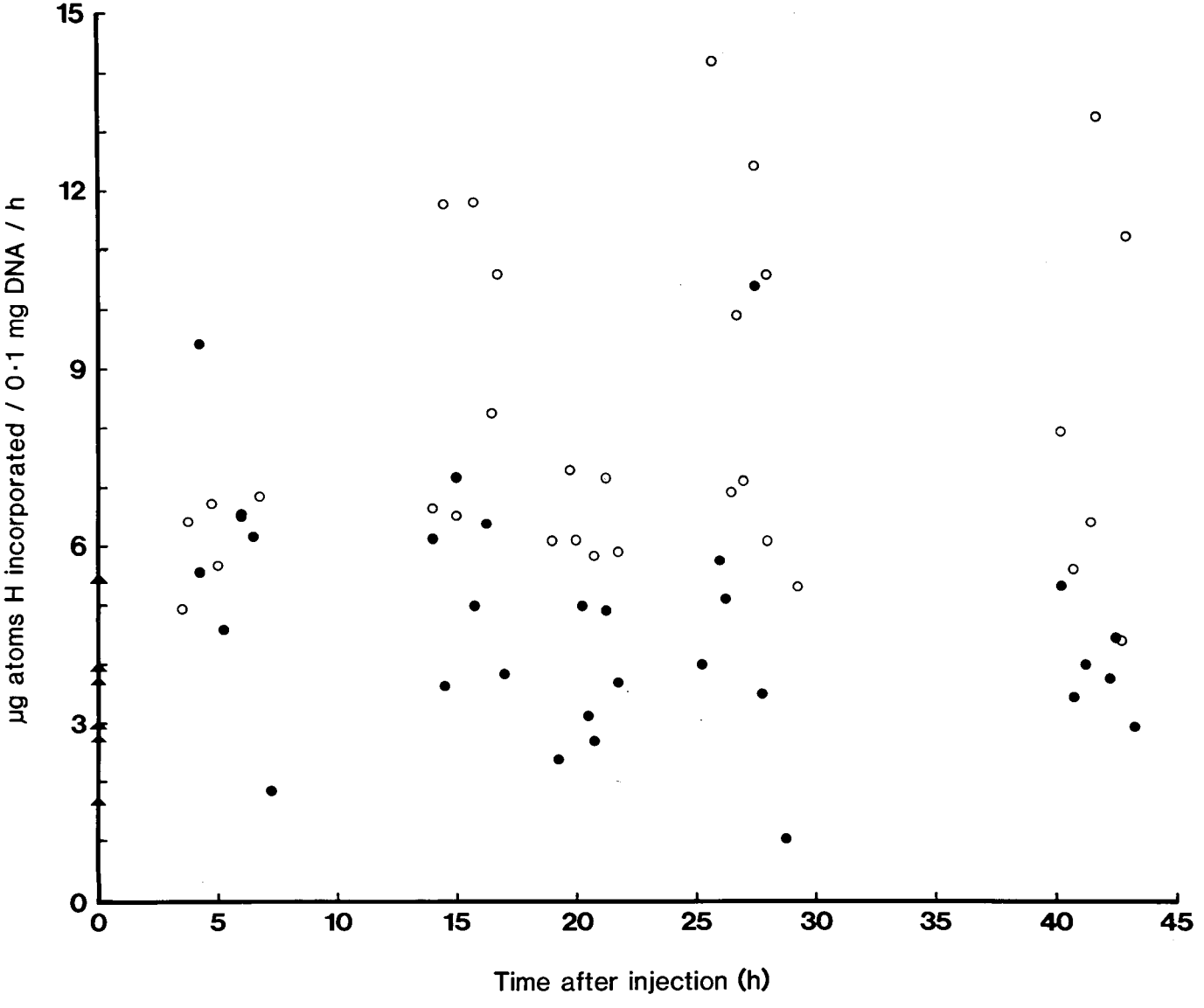

15
12
9
6
3
0
0
5
10
15
20
25
30
35
40
45
µg atoms H incorporated / 0·1 mg DNA / h
Time after injection (h)

TABLE 10

The incorporation of tritium from ^3H_2O into total lipid by liver slices from control and oestrogen-treated male chicks at varying times after injection

Incorporation of tritium from ^3H_2O		Untreated	Time after a single injection (h)						Multiple injections	
			$3\frac{1}{2}$ - $7\frac{1}{4}$	14 - 17	19 - $21\frac{3}{4}$	$25\frac{1}{4}$ - $28\frac{3}{4}$	$29\frac{1}{4}$ - $31\frac{1}{2}$	$40\frac{1}{4}$ - $43\frac{1}{4}$	x 7	x 11
µg atoms H/ 100 mg liver/h	E	9·6 ± 1·4 (8)	17·6 ± 1·5 (6)	20·8 ± 2·7 (6)	15·9 ± 1·5 (6)	20·6 ± 2·4 (7)	11·8 ± 1·2 (2)	18·8 ± 3·2 (6)	8·6 (1)	7·0 (1)
	C		16·3 ± 2·8 (6)	15·7 ± 2·1 (6)	11·7 ± 2·0 (6)	15·8 ± 3·3 (7)	7·7 (1)	12·1 ± 1·5 (6)	12·2 (1)	11·0 (1)
µg atoms H/ liver/min	E	16·5 ± 2·2 (8)	28·3 ± 1·5 (6)	46·5 ± 5·1 (6) ++	40·0 ± 3·9 (6) Δ	48·4 ± 3·7 (7) ++	23·3 ± 12·2 (2)	53·5 ± 9·0 (6) ++	12·0 (1)	13·7 (1)
	C		26·8 ± 4·2 (6)	26·6 ± 2·9 (6)	20·3 ± 2·6 (6)	25·5 ± 5·7 (7)	5·0 (1)	22·3 ± 2·1 (6)	12·1 (1)	15·1 (1)
µg atoms H/ 0·1 mg DNA/h	E	3·3 ± 0·4 (8)	6·2 ± 0·3 (6)	9·3 ± 1·0 (6) ++	6·4 ± 0·3 (6) ▲	9·6 ± 1·2 (7) †	5·3 (1)	8·1 ± 1·4 (6) ‡	N.D.	N.D.
	C		5·7 ± 1·0 (6)	5·3 ± 0·6 (6)	3·6 ± 0·5 (6)	5·0 ± 1·3 (6)	N.D.	4·0 ± 0·3 (6)	N.D.	N.D.

See legend of Fig. 17 for experimental details.

Incubations were performed in triplicate, and the results are the average values for a single chick or the means (± S.E.M.) of the average values obtained from 2 - 8 chicks. The number of birds involved in each group is given in parentheses.

E = values for oestrogen-treated chicks

C = values for control chicks

N.D. = not determined

Data were analyzed statistically by Student's 't' test, and levels of statistical significance are presented with respect to values for corresponding control chicks.

†	significant at $P < 0{\cdot}05$
‡	significant at $P < 0{\cdot}02$
††	significant at $P < 0{\cdot}01$
Δ	significant at $P < 0{\cdot}002$
▲	significant at $P < 0{\cdot}001$

In Fig. 17 and Table 10, the results of the experiments have been expressed as μg atoms H incorporated/100 mg liver/hour. As was found in the acetate incorporation studies, there was a lot of variability among birds. Statistical analysis of the results showed that none of the values for the oestrogen-treated groups of chicks differed significantly from the values for their corresponding control groups (Table 10). However, for all groups receiving a single injection, the mean value for the oestrogen-treated chicks was greater than the mean value for the control chicks in each time group. In addition, it is of interest to note that the results for the untreated chicks were found to be significantly lower than the results for the $3\frac{1}{2}$ - $7\frac{1}{4}$ h and 14 - 17 h control groups ($P < 0{\cdot}05$). The mean values for the $3\frac{1}{2}$ - $7\frac{1}{4}$ h and 14 - 17 h control groups were approximately 70% and 64% higher, respectively, than the mean value for the untreated group of chicks. Insufficient data prevented any sort of statistical analysis or deductions being made about the effect of multiple injections on tritium incorporation from $^{3}H_2O$ into total lipid by liver slices.

In Fig. 18 and Table 10, the results of the experiments have been expressed as μg atoms H incorporated/liver/minute. Between approximately 14 and 43 hours after a single oestrogen injection, the values for the incorporation of tritium from $^{3}H_2O$ into total lipid appeared to be generally greater than the values for untreated and control chicks (Fig. 18). Statistical analysis of the results showed that the values for the oestrogen-treated chicks in the 14 - 17 h, 19 - $21\frac{3}{4}$ h, $25\frac{1}{4}$ - $28\frac{3}{4}$ h and $40\frac{1}{4}$ - $43\frac{1}{4}$ h groups were significantly greater than the values for their corresponding control chicks with probabilities (P) of less than 0·01, 0·002, 0·01 and 0·01 respectively. The mean values for these oestrogen-treated groups of birds were approximately 1·75-fold, 1·97-fold, 1·90-fold and 2·40-fold greater, respectively, than the mean values for

their corresponding control groups. The results for the untreated chicks were found to be significantly lower than the results for the $3\frac{1}{2}$ - $7\frac{1}{4}$ h control group ($P < 0{\cdot}05$) and the 14 - 17 h control group ($P < 0{\cdot}02$). The mean values for the $3\frac{1}{2}$ - $7\frac{1}{4}$ h and 14 - 17 h control groups were approximately 1·62-fold and 1·61-fold higher, respectively, than the mean value for the untreated group of chicks. Insufficient data prevented any analysis of the results from the birds that had received multiple injections.

In Fig. 19 and Table 10, the results of the experiments have been expressed as µg atoms H incorporated/0·1 mg liver DNA/hour. Between approximately 14 and 43 hours after a single oestrogen injection, the values for the incorporation of tritium from ^3H_2O into total lipid had a tendency to be greater than the values for untreated and control chicks (Fig. 19). Statistical analysis of the results showed that the values for the oestrogen-treated chicks in the 14 - 17 h, 19 - $21\frac{3}{4}$ h, $25\frac{1}{4}$ - $28\frac{3}{4}$ h and $40\frac{1}{4}$ - $43\frac{1}{4}$ h groups were significantly greater than the results for their corresponding control groups with probabilities (P) of less than 0·01, 0·001, 0·05 and 0·02 respectively. The mean values for these oestrogen-treated groups of chicks were approximately 1·76-fold, 1·78-fold, 1·92-fold and 2·03-fold greater, respectively, than the mean values for their corresponding control groups. The results for the group of untreated chicks were significantly lower than the results for the $3\frac{1}{2}$ - $7\frac{1}{4}$ h control group ($P < 0{\cdot}05$) and the 14 - 17 h control group ($P < 0{\cdot}01$), the mean values for these control groups being 1·73-fold and 1·61-fold higher, respectively, than the mean value for untreated chicks.

The results of the saponification and t.l.c. analyses of lipid extracts from control and oestrogenized chick liver slices, after incubation with ^3H_2O, are presented in Tables 8 and 9. The recovery of

radioactivity in the saponification procedure was routinely about 80% (Table 8). On average, for samples from control chicks, about 4% of this was attributable to non-saponifiable lipids and about 96% to fatty acids derived from complex lipids. For samples from oestrogen-treated chicks, on average about 6·5% of the recovered radioactivity was attributable to non-saponifiable lipids and about 93·5% to fatty acids derived from complex lipids. These percentages of the recovered radioactivity in non-saponifiable lipids and fatty acids of complex lipids were not found to differ significantly for control and oestrogen-treated chicks.

The recovery of radioactivity from t.l.c. plates in the $^{3}H_{2}O$ incorporation studies was routinely about 65% (Table 9). For lipid extracts from control chick liver slices, about 86·9% of the label was located in triacylglycerol, with about 6·1% being located at the origin (phospholipid), 0·2% in monoacylglycerol, 4·4% in diacylglycerol and cholesterol, and about 0·4% in free fatty acids. A similar distribution of the label in the lipid classes was observed for lipid extracts from liver slices of oestrogenized chicks, with about 87·2% of the label being located in triacylglycerol, 6·0% at the origin (phospholipid), 0·2% in monoacylglycerol, 4·1% in diacylglycerol and cholesterol, and 0·4% in free fatty acids. There were no significant differences between control and oestrogen-treated liver lipid extracts in the percentages of the recovered radioactivity in the lipid classes.

3. [9,10-^{3}H] Palmitate incorporation studies

The capacity of liver slices from untreated, oestrogen-treated and control chicks to incorporate pre-formed fatty acids into complex lipids was investigated by measuring the incorporation of [9,10-^{3}H] palmitate into neutral lipids. From the incorporation studies using [1-^{14}C]

acetate and $^{3}H_2O$ it was evident that the main product of lipogenesis was triacylglycerol, since t.l.c. analyses showed that the majority of the label was recovered in the triacylglycerol fraction in each case. The lipid extracts from [9,10-^{3}H] palmitate incubations were, therefore, subjected to t.l.c. analysis, and the triacylglycerol fractions were isolated for counting. Initial experiments were carried out to establish a suitable incubation time and palmitate concentration for studying the incorporation of [9,10-^{3}H] palmitate into triacylglycerol by liver slices from control and oestrogen-treated chicks. Oestrogenized chicks received a single intramuscular injection of 1 mg 17β-oestradiol in propane-1,2-diol/100 g body weight approximately 48 hours before death. Control chicks received an equivalent volume of propane-1,2-diol only.

The effect of incubation time on the incorporation of [9,10-^{3}H] palmitate into triacylglycerol was studied using a palmitate concentration of 0·65 mM, since Hawkins & Heald [236] adopted a similar concentration in their studies. The results of this investigation are presented in Fig. 20. The data do not show the existence of an initial lag phase, and the results for liver slices from both control and oestrogen-treated chicks suggested that the incorporation of [9,10-^{3}H] palmitate was linear for at least 90 minutes. A 60 minute incubation period was adopted in all subsequent incubations since this incubation time had been used for the [1-^{14}C] acetate and $^{3}H_2O$ incorporation studies and it seemed adequate.

The effect of palmitate concentration on the incorporation of [9,10-^{3}H] palmitate into triacylglycerol by chick liver slices, during a 60 minute incubation, was investigated over a palmitate concentration range of 0·162 to 1·293 mM. The results of this investigation are presented in Fig. 21, from which it can be seen that incorporation increased

FIGURE 20

The effect of incubation time on the incorporation of 0·65 mM-[9,10-^{3}H] palmitate into triacylglycerol by liver slices from control and oestrogen-treated chicks

Each oestrogen-treated chick received a single intramuscular injection of 1 mg 17β-oestradiol in propane-1,2-diol/100 g body weight 48 hours before death. Control birds received an equivalent volume of propane-1,2-diol only.

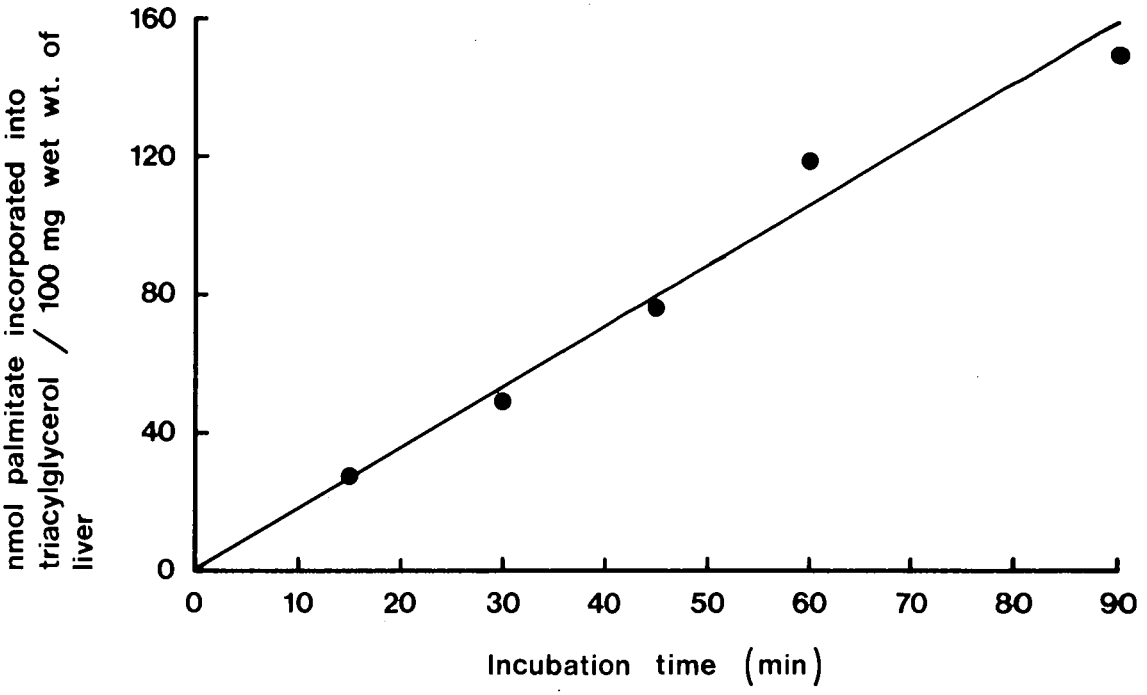
Chick liver slices (150 ± 10 mg, wet wt.) were incubated at 40°C in 3 ml avian bicarbonate buffer, pH 7·4, containing 12 mM-β-D(-)-fructose and 0·65 mM-[9,10-^{3}H] palmitate (1·95 μCi). Total lipid was extracted at the times indicated, and lipid classes were separated by t.l.c. as described in the text.

(a) values for control chicks

(b) values for oestrogen-treated chicks

Each value represents the average of single determinations for 2 birds.

(a)


nmol palmitate incorporated into triacylglycerol / 100 mg wet wt. of liver
160
120
80
40
0
0
10
20
30
40
50
60
70
80
90
Incubation time (min)

(b)

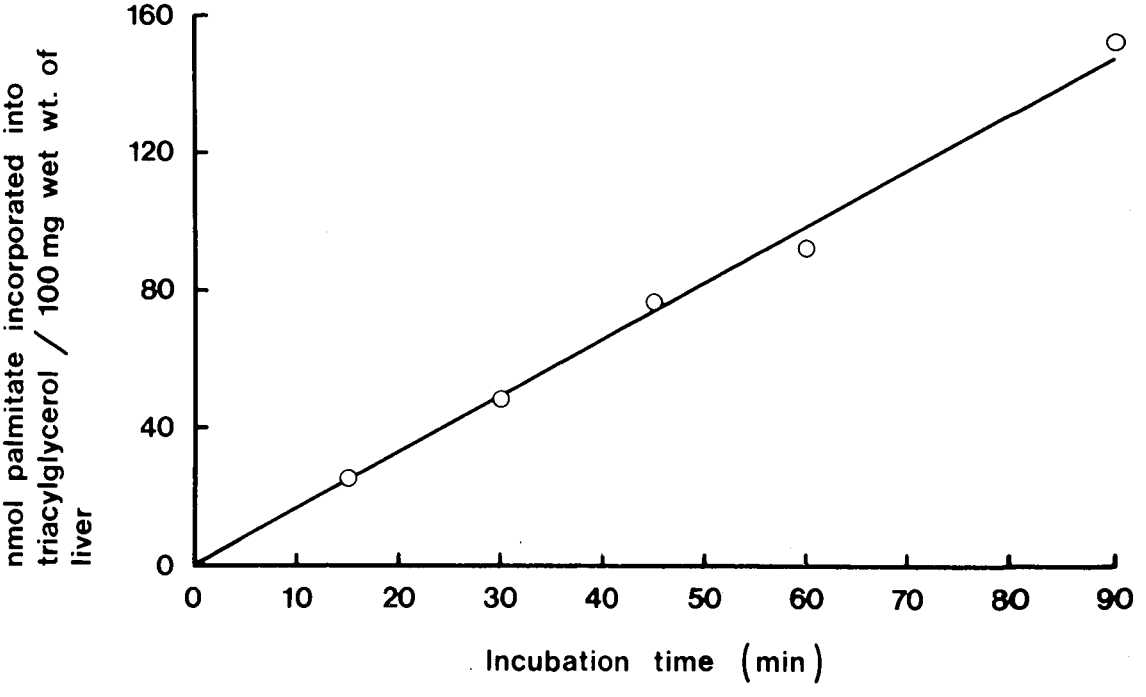

nmol palmitate incorporated into triacylglycerol / 100 mg wet wt. of liver
160
120
80
40
0
0
10
20
30
40
50
60
70
80
90
Incubation time (min)

FIGURE 21

The effect of palmitate concentration on the incorporation of [9,10-^{3}H] palmitate into triacylglycerol by liver slices from control and oestrogen-treated chicks

Each oestrogen-treated chick received a single intramuscular injection of 1 mg 17 β-oestradiol in propane-1,2-diol/100 g body weight 48 hours before death. Control chicks received an equivalent volume of propane-1,2-diol only.

Chick liver slices (150 ± 10 mg, wet wt.) were incubated at 40°C in 3 ml avian bicarbonate buffer, pH 7·4, containing 12 mM-β-D(-)-fructose and 0·162 to 1·293 mM-[9,10-^{3}H] palmitate (3 μCi/μmole). Total lipid was extracted after a 60 minute incubation, and lipid classes were separated by t.l.c. as described in the text.

● values for control chicks

○ values for oestrogen-treated chicks

Each value represents the average of single determinations for 2 birds.

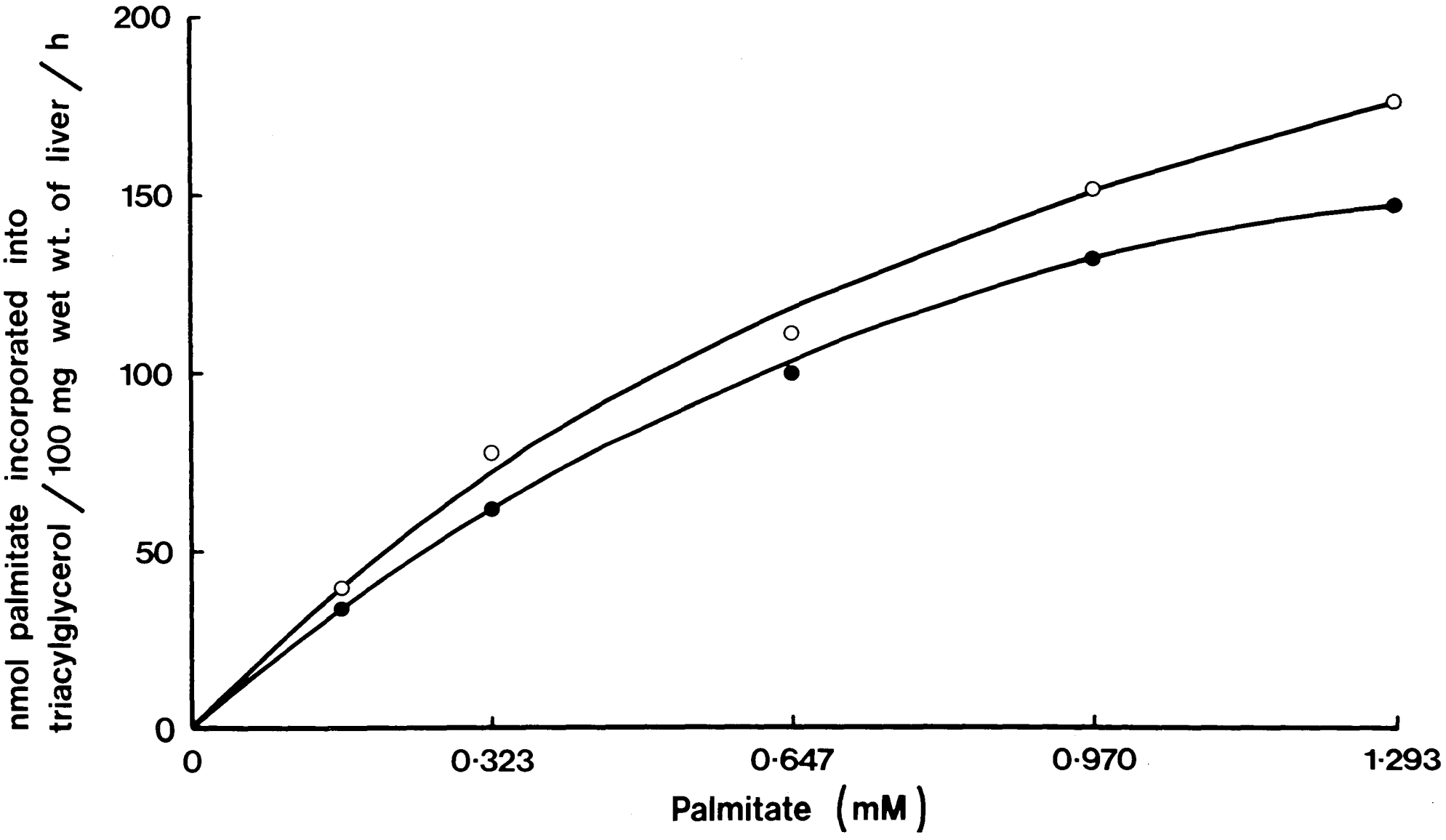

nmol palmitate incorporated into triacylglycerol / 100 mg wet wt. of liver / h
200
150
100
50
0
0
0·323
0·647
0·970
1·293
Palmitate (mM)

FIGURE 22

The incorporation of 0·65 mM-[9,10-^{3}H] palmitate into triacylglycerol by liver slices (nmol palmitate incorporated/100 mg liver/h) from control and oestrogen-treated male chicks at varying times after injection

Oestrogen-treated chicks received an intramuscular injection of 1 mg 17 β-oestradiol in propane-1,2-diol/100 g body weight. Control chicks received an equivalent volume of propane-1,2-diol only. Untreated chicks were also included in the experiments. At the indicated times after injection birds were sacrificed and liver slices were prepared.

Liver slices (150 ± 10 mg, wet wt.) were incubated at 40°C in 3 ml avian bicarbonate buffer, pH 7·4, containing 12 mM-β-D(-)-fructose and 0·65 mM-[9,10-^{3}H] palmitate (1·95 μCi). Total lipid was extracted after a 60 minute incubation, and lipid classes were separated by t.l.c. as described in the text.

▲ values for untreated chicks

● values for control chicks

○ values for oestrogen-treated chicks

Each value represents the mean result from 3 incubations for a single bird.

Chicks were aged 4 – 4½ weeks.

with increasing palmitate concentration in a curvilinear fashion. A palmitate concentration of approximately 0·65 mM was chosen for use in all subsequent incubations, since very high palmitate concentrations would have been needed to completely saturate the system, and such high concentrations would have been very difficult, if not impossible, to prepare. Plasma free fatty acid levels have been presented in the literature, ranging from 0·2 to 0·5 mM in the immature domestic fowl to 1 to 3 mM in the mature laying hen, whilst at the point of lay levels can reach 4·5 mM [57, 127, 236]. Hawkins & Heald [236] injected 11-week old pullets with 2 mg oestradiol monobenzoate on alternate days for 7 days, and obtained plasma free fatty acid levels on the 8th day of 7·2 - 7·7 mM, compared with 0·5 - 0·9 mM for untreated pullets. These workers also showed that the incorporation of palmitate into neutral lipids by liver slices from immature and laying domestic fowl increased linearly with increasing palmitate concentration to at least 2·13 mM.

Experiments were carried out to investigate the incorporation of [9,10-^{3}H] palmitate into triacylglycerol by liver slices from untreated chicks, chicks treated with a single intramuscular injection of 1 mg 17 β-oestradiol in propane-1,2-diol/100 g body weight, and chicks injected with an equivalent volume of propane-1,2-diol only. Birds were sacrificed at varying times after injection, and liver slice incubations were performed as described in the Methods section. The results of these experiments are presented in Figs. 22 - 24 and in Table 11. In Table 11, the chicks have been allocated to groups dependent upon treatment and upon the time of death after a single injection ($3\frac{3}{4}$ - $51\frac{3}{4}$ h). The results for each group of oestrogen-treated chicks have been compared with the results for the corresponding group of control chicks. The data were analyzed statistically by Student's 't' test, and levels of statistical significance are presented in the table and text where appropriate.

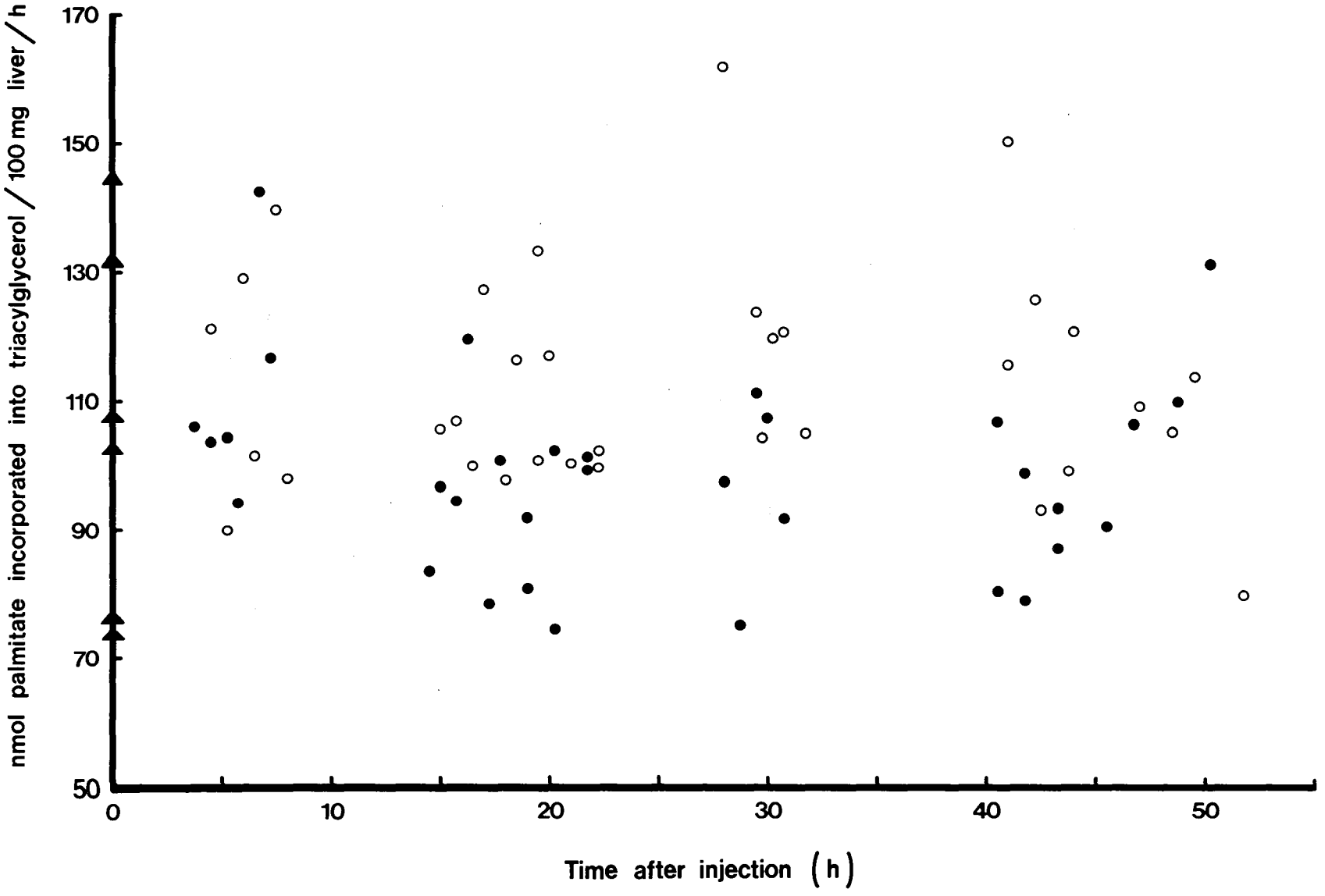

nmol palmitate incorporated into triacylglycerol / 100 mg liver / h
170
150
130
110
90
70
50
0
10
20
30
40
50
Time after injection (h)

FIGURE 23

The incorporation of 0·65 mM-[9,10-^{3}H] palmitate into triacylglycerol by liver slices (nmol palmitate incorporated/liver/min) from control and oestrogen-treated male chicks at varying times after injection

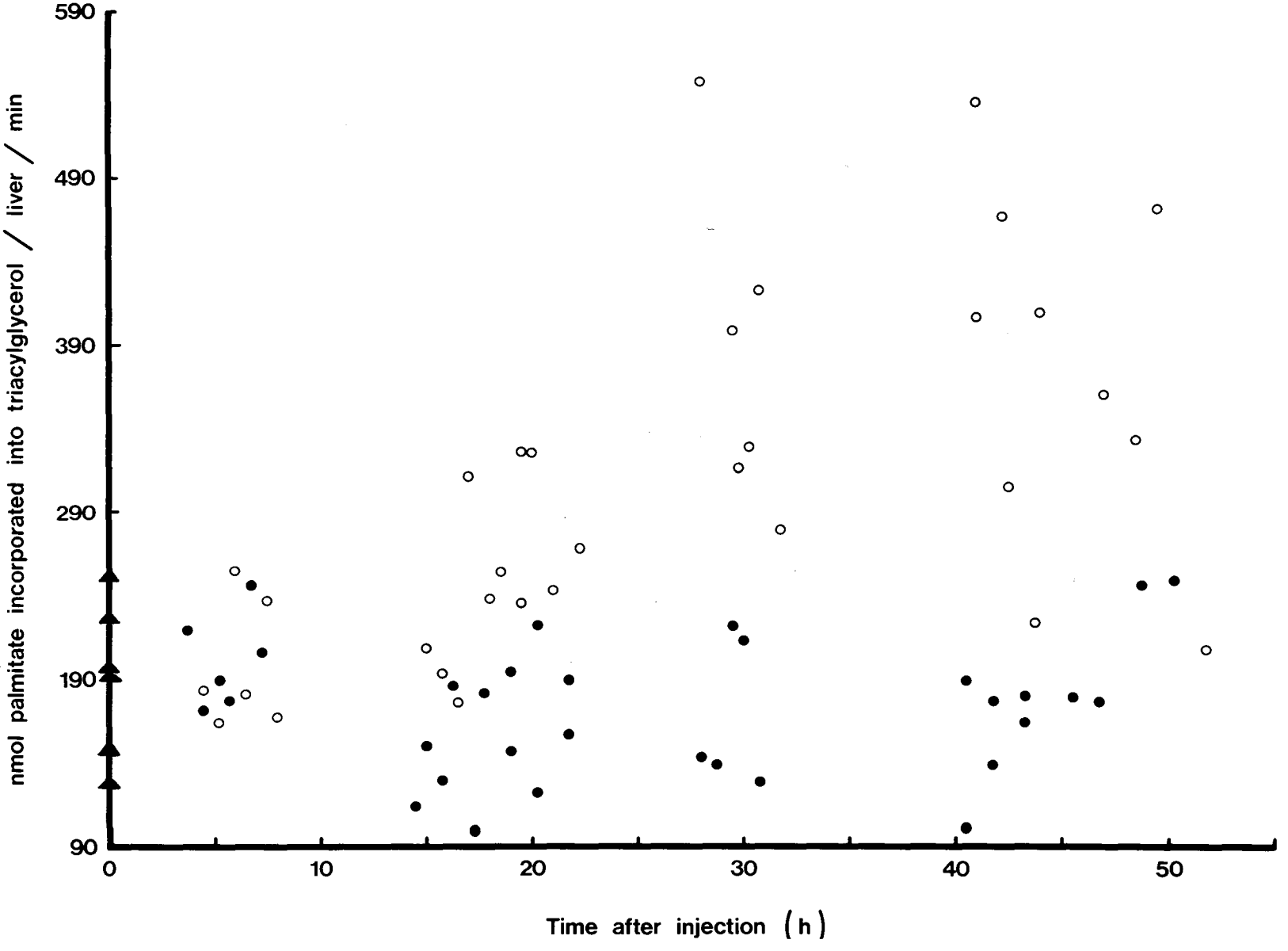
See legend of Fig. 22 for experimental details.

▲ values for untreated chicks

● values for control chicks

○ values for oestrogen-treated chicks

Each value represents the mean result from 3 incubations for a single bird.


590
490
390
290
190
90
0
10
20
30
40
50
nmol palmitate incorporated into triacylglycerol / liver / min
Time after injection (h)

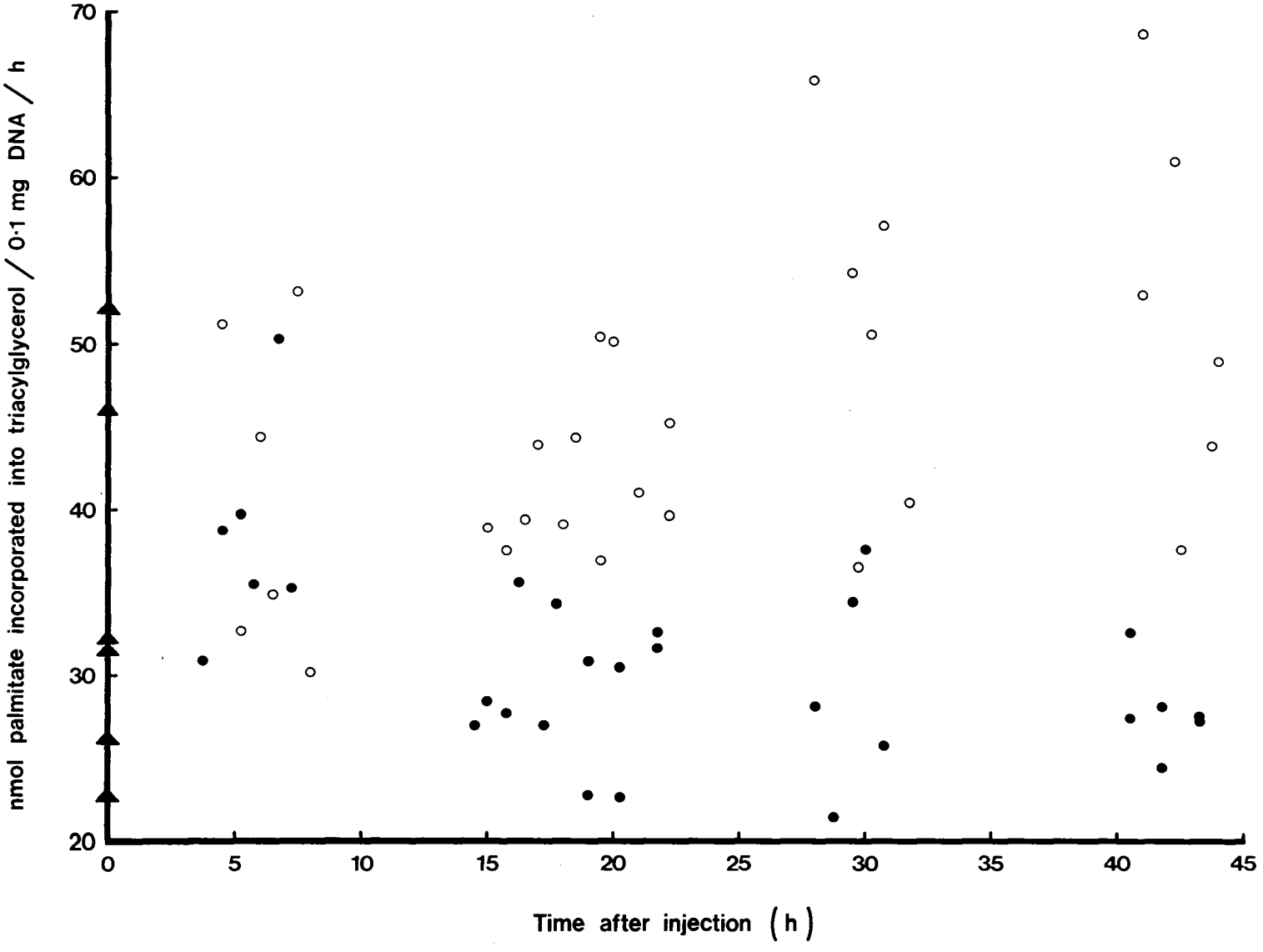

nmol palmitate incorporated into triacylglycerol / 0·1 mg DNA / h
70
60
50
40
30
20
0
5
10
15
20
25
30
35
40
45
Time after injection (h)

TABLE 11

The incorporation of 0·65 mM-[9,10-^{3}H] palmitate into triacylglycerol by liver slices from control and oestrogen-treated male chicks at varying times after injection

Incorporation of [9,10-^{3}H] palmitate		Untreated	Time after injection (h)					
			3¾ - 8	14½ - 18½	19 - 22¼	28 - 31¾	40½ - 44	45½ - 51¾
nmol palmitate/ 100 mg liver/h	E	106·2 ± 11·6 (6)	113·2 ± 8·0 (6)	108·8 ± 4·5 (6)	108·8 ± 5·6 (6) †	122·3 ± 8·6 (6) †	117·1 ± 8·3 (6) ‡	101·6 ± 7·5 (4)
	C		111·2 ± 6·9 (6)	95·5 ± 5·9 (6)	91·6 ± 4·7 (6)	96·4 ± 6·4 (5)	90·8 ± 4·4 (6)	109·2 ± 8·3 (4)
nmol palmitate/ liver/min	E	189·7 ± 19·2 (6)	197·6 ± 15·8 (6)	229·8 ± 20·1 (6) ††	277·6 ± 16·1 (6) ▲	381·8 ± 39·6 (6) Δ	389·9 ± 45·5 (6) ▲	341·9 ± 54·0 (4)
	C		201·6 ± 11·6 (6)	142·6 ± 14·6 (6)	171·4 ± 15·1 (6)	168·7 ± 20·0 (5)	157·7 ± 13·4 (6)	212·0 ± 20·2 (4)
nmol palmitate/ 0·1 mg DNA/h	E	35·2 ± 4·7 (6)	41·1 ± 4·0 (6)	40·6 ± 1·2 (6) ▲	43·9 ± 2·3 (6) ▲	50·8 ± 4·4 (6) ††	52·1 ± 4·6 (6) ▲	N.D.
	C		38·4 ± 2·7 (6)	30·0 ± 1·6 (6)	28·5 ± 1·9 (6)	29·5 ± 2·9 (5)	27·8 ± 1·1 (6)	N.D.

See legend of Fig. 22 for experimental details.

Incubations were performed in triplicate and the incubation products from a bird were combined to give a single value. The results are the means (± S.E.M.) of values obtained from 4 - 6 chicks. The number of birds involved in each group is given in parentheses.

E = values for oestrogen-treated chicks

C = values for control chicks

N.D. = not determined

Data were analyzed statistically by Student's 't' test, and levels of statistical significance are presented with respect to values for corresponding control chicks.

† significant at $P < 0{\cdot}05$

‡ significant at $P < 0{\cdot}02$

†† significant at $P < 0{\cdot}01$

Δ significant at $P < 0{\cdot}002$

▲ significant at $P < 0{\cdot}001$

In Fig. 22 and Table 11, the results of the experiments have been expressed as nmoles palmitate incorporated into triacylglycerol/100 mg liver/hour. From Fig. 22 it is difficult to detect differences between the results for the untreated, control and oestrogen-treated chicks, although at times later than 15 hours after injection the values for the oestrogen-treated chicks showed a tendency to be greater than corresponding control values. Statistical analysis of the results showed that the values for the oestrogen-treated chicks in the 19 - 22¼ h, 28 - 31¾ h and 40½ - 44 h groups were significantly greater than the results for the corresponding control chicks with probabilities (P) of less than 0·05, 0·05 and 0·02 respectively. The mean values for the oestrogen-treated chicks in the 19 - 22¼ h, 28 - 31¾h and 40½ - 44 h groups were approximately 19%, 27% and 29% higher, respectively, than the mean values for their corresponding control groups. The values for the untreated chicks did not differ significantly from any of the values for control chicks.

In Fig. 23 and Table 11, the results of the experiments have been expressed as nmoles palmitate incorporated into triacylglycerol/liver/minute. From Fig. 23 it is evident that between approximately 17 and 49½ hours after a single oestrogen injection the palmitate incorporation values were generally greater than values for control and untreated chicks. The results for the oestrogen-treated chicks in the 14½ - 18½ h, 19 - 22¼ h, 28 - 31¾ h and 40½ - 44 h groups were significantly greater than the results for the corresponding control chicks with probabilities (P) of less than 0·01, 0·001, 0·002 and 0·001 respectively. The mean values for the oestrogen-treated chicks in the 14½ - 18½ h, 19 - 22¼ h, 28 - 31¾ h and 40½ - 44 h groups were approximately 1·61-fold, 1·62-fold, 2·26-fold and 2·47-fold greater, respectively, than the mean values for their corresponding control groups. The values for the

untreated birds were not significantly different from any of the values for control birds.

In Fig. 24 and Table 11, the results of the experiments have been expressed as nmoles palmitate incorporated into triacylglycerol/0·1 mg liver DNA/hour. Between approximately 15 and 44 hours after a single oestrogen injection, the palmitate incorporation values were generally greater than corresponding control values (Fig. 24). Statistical analysis of the data showed that the results for the oestrogen-treated chicks in the $14\frac{1}{2}$ - $18\frac{1}{2}$ h, 19 - $22\frac{1}{4}$ h, 28 - $31\frac{3}{4}$ h and $40\frac{1}{2}$ - 44 h groups were significantly greater than the results for the corresponding control birds with probabilities (P) of less than 0·001, 0·001, 0·01 and 0·001 respectively. The mean values for the oestrogen-treated chicks in the $14\frac{1}{2}$ - $18\frac{1}{2}$ h, 19 - $22\frac{1}{4}$ h, 28 - $31\frac{3}{4}$ h and $40\frac{1}{2}$ - 44 h groups were approximately 35%, 54%, 72% and 87% greater, respectively, than the mean values for their corresponding control groups. The values for the untreated chicks did not differ significantly from any of the values for control chicks.

In these [9,10-^{3}H] palmitate incorporation studies only the iodine-staining areas on the t.l.c. plates were removed and counted, and the recovery of radioactivity was generally about 70%. The distribution of the label in the lipid classes is presented in Table 12. For lipid extracts from control and untreated chick liver slices, about 92·3% of the recovered radioactivity was attributable to triacylglycerol, with approximately 3·3% of the label being located as free fatty acid, 2·7% at the origin (phospholipid) and 1·3% in diacylglycerol and cholesterol. A similar distribution of the label in the lipid classes was observed for lipid extracts from liver slices of oestrogen-treated chicks, with about 92·9% of the label being located in triacylglycerol, about 2·8% as free fatty acid, 2·6% at the origin (phospholipid) and about 1·3% in

Values are the means (± S.E.M.) of the results for 34 - 65 chicks.

The number of observations involved in each group is given in parentheses.

Oestrogen-treated chicks received a single intramuscular injection of 1 mg 17β-oestradiol in propane-1,2-diol/100 g body weight. Control chicks received an equivalent volume of propane-1,2-diol only.

At various times after injection, chicks were sacrificed and liver slices were prepared. Liver slices (150 ± 10 mg, wet wt.) were incubated in 3 ml avian bicarbonate buffer, pH 7·4, containing 12 mM-β-D(-)-fructose and 0·65 mM-[9,10-^{3}H] palmitate (1·95 μCi). Total lipid was extracted after a 60 minute incubation at 40°C, and lipid classes were separated by t.l.c. as described in the text.

Data were analyzed statistically by Student's 't' test, and levels of statistical significance are presented where appropriate.

†† significant at $P < 0{\cdot}01$

diacylglycerol and cholesterol. A significantly greater percentage of the recovered radioactivity was located in free fatty acid after t.l.c. analysis of lipid extracts from control and untreated chick liver slices than was obtained for lipid extracts from oestrogen-treated chick liver slices ($P < 0{\cdot}01$). In addition, a significantly greater percentage of the recovered radioactivity was located in triacylglycerol in lipid extracts from oestrogen-treated chick liver slices than in lipid extracts from control and untreated chick liver slices ($P < 0{\cdot}01$).

DISCUSSION

1. [1-^{14}C] Acetate incorporation studies

Acetate thiokinase (EC 6.2.1.1)* is responsible for the conversion of [1-^{14}C] acetate to labelled acetyl-CoA, and the activity and location of this enzyme are, therefore, extremely important when acetate is being used as a precursor for lipids. The ^{14}C-labelled acetyl-CoA can enter the Krebs cycle, or be converted to fatty acids via the actions of acetyl-CoA carboxylase (EC 6.4.1.2) and fatty acid synthetase.

The incorporation of labelled acetyl-CoA into fatty acids will, therefore, be dependent upon the rate of entry of labelled acetyl-CoA into the Krebs cycle. In addition to the acetyl-CoA formed from acetate, acetyl-CoA can also be formed from other precursors such as pyruvate, and by the degradation of fatty acids and amino acids. Hence, the labelled acetyl-CoA pool formed from [1-^{14}C] acetate will be diluted by unlabelled acetyl-CoA formed from these other precursors. Acetate is a poor physiological substrate for the production of acetyl-CoA in chick liver, but it was chosen for use in the present

* Acetyl-CoA synthetase

studies of de novo lipogenesis because its conversion to acetyl-CoA involves only one metabolic step, and it does not readily enter other metabolic pathways.

De novo lipogenesis from [1-^{14}C] acetate by livers of control and oestrogen-treated male chicks was studied by incubating liver slices in avian bicarbonate buffer for 1 hour at 40°C, in the presence of 10 mM-sodium [1-^{14}C] acetate. These conditions resemble those adopted by other workers in similar studies with chick liver preparations. All incubations were performed at 40°C, since this temperature closely approximates chick body temperature and has been widely used in studies of chick metabolism by Goodridge (34). Leveille (33) employed 38°C, and Kudzma et al. (113) and Weiss et al. (237) employed 37°C as the incubation temperature during chick liver slice studies. On the other hand, Hawkins & Heald (236) and Duncan (238) adopted temperatures of 41·3°C and 41·5°C, respectively, which are within the actual range of body temperature of the domestic fowl (391). The concentration of sodium [1-^{14}C] acetate found to produce maximum incorporation into lipid in the present study was 10 mM, which is the same as that reported by Leveille (239) and suggested by Goodridge (276). The data presented in Fig. 12 show that inhibition of incorporation occurs with acetate concentrations greater than 10 mM. In the present investigation, liver slices synthesized lipids from 10 mM-sodium [1-^{14}C] acetate in a linear fashion for 1 hour. This is a somewhat shorter period than observed by several other workers, who have reported linear incorporation of labelled acetate into lipid by liver slices for up to 3 hours (33, 113, 237 - 239, 276, 392). The initial lag phase was undoubtedly the result of delays in the diffusion of the labelled substrate into the cells and in the equilibration of the labelled acetyl-CoA with the unlabelled pool of acetyl-CoA, and also of the time taken by the tissue to attain the correct temperature.

The results of the acetate incorporation studies were expressed as nmoles acetate incorporated into lipid/100 mg liver/hour, as nmoles acetate incorporated into lipid/liver/minute and as nmoles acetate incorporated into lipid/0·1 mg liver DNA/hour (Figs. 13 - 15, Tables 6 and 7). Treating male chicks with oestrogen results in an increase in the size and weight of the liver, as shown in Chapter 2. It was, therefore, considered important to determine the total hepatic capacity for lipogenesis in each case, so as to ascertain whether variations in lipogenic capacity were the result of different liver sizes. This liver enlargement associated with oestrogen treatment is considered to be predominantly caused by cell expansion, as a result of water uptake and accumulation of lipid, with some cell division (242). Consequently, a unit wet weight of liver from an oestrogen-treated chick would undoubtedly contain fewer cells than an equivalent unit wet weight of liver from a control or untreated chick. It was considered important, therefore, to express incorporation results on a cellular basis, which was accomplished by determining the DNA content of the liver in each case, and by expressing the results as rates of incorporation per unit weight of DNA. By presenting the data in these various ways, it was hoped that any differences in the capacities of the livers to incorporate [1-^{14}C] acetate into total lipid, either at the cellular level or on the level of the whole organ, would become apparent.

When acetate incorporation into total lipid was expressed as nmoles acetate/100 mg liver/hour (Fig. 13, Table 6), a significant difference between the results for oestrogen-treated and control birds was obtained at 17 - 21¾ hours after injection ($P < 0{\cdot}01$). The mean value for the oestrogen-treated chicks in this group was approximately 2-fold greater than the mean value for the control chicks. In the absence of an appropriate control group, the results for the oestrogen-treated birds

in the 30 - 31 h group were compared with those for the control birds in the other groups and were found to be significantly greater than the results for the $12\frac{1}{4}$ - $14\frac{1}{4}$ h control group ($P < 0{\cdot}05$). However, these control groups are not directly comparable with the 30 - 31 h oestrogen-treated group and, therefore, such comparisons cannot be considered to be valid . It should be noted at this stage that the 100 mg samples of liver from the oestrogen-treated chicks would be expected to contain fewer cells than the 100 mg samples of liver from the control chicks.

When the acetate incorporation results were expressed as nmoles acetate incorporated/liver/minute (Fig. 14, Table 6), the results for the oestrogen-treated chicks in the 17 - $21\frac{3}{4}$ h group were, once again, significantly greater than the results for the corresponding control chicks ($P < 0{\cdot}002$). In this case, the mean value for the oestrogen-treated chicks was more than 3-fold greater than the mean value for the control group. Hence, expressing the results on a total organ basis increased the difference between oestrogenized and control values that had been revealed when the results were expressed per 100 mg liver. Therefore, at this time after injection the lipogenic capacity of a unit weight of oestrogen-treated liver appears to be greater than that of an equivalent weight of control liver, and in addition, the lipogenic capacity of the oestrogenized liver is increased still further by virtue of an increase in liver size. Expression of the acetate incorporation data as nmoles acetate incorporated/liver/minute (Fig. 14) clearly shows a greater divergence between the oestrogenized and control values after about 17 hours post-injection than is evident from Fig. 13 in which incorporation was expressed per 100 mg liver. The results obtained for the oestrogenized chicks in the 30 - 31 h group were found to be significantly greater than the control values in all the other groups. Had control birds been included in the 30 - 31 h group, it would seem likely

that the results for the oestrogenized birds would have been significantly greater than these too, but this has not been shown. Expressing the data as total organ incorporation increased the differences between the oestrogenized birds in the 30 - 31 h group and control birds that had been evident when the results were expressed per 100 mg liver.

Rosebrough et al. (251) have also observed a greater difference between the livers of oestrogen-treated and control birds in their lipogenic capacity from acetate when results were expressed on a whole liver basis rather than a unit liver weight basis. These workers injected Large White turkey hens with 100 mg 17 β-oestradiol (a 25 mg dose followed by a 75 mg dose one week later) in corn oil, or with corn oil only. One week after the last dose hens were killed, livers were isolated and liver slices were prepared. The liver slices were incubated with sodium [1-^{14}C] acetate and incorporation of [1-^{14}C] acetate into the fatty acids of complex lipids was determined. When results were expressed as nmoles acetate incorporated/100 mg liver/2 hours, hens treated with 17 β-oestradiol synthesized fatty acids at a 1·5-fold greater rate than the control hens. When results were expressed on a whole liver basis, hens treated with 17 β-oestradiol synthesized fatty acids at a 2·5-fold greater rate than the control hens.

Expression of the acetate incorporation data on a cellular basis also reveals divergence between values for oestrogenized and control chicks (Fig. 15). From Table 6, it can be seen that the results for oestrogen-treated chicks were significantly greater than those for control chicks at 17 - 21¾ and 48¾ - 55½ hours after injection, with the greatest difference being observed in the 17 - 21¾ h group ($P < 0{\cdot}01$). The mean value for this group of oestrogen-treated chicks was about 2·76-fold greater than the control value, indicating that the liver cells of the oestrogen-treated birds had a greater capacity to incor-

porate [1-^{14}C] acetate into lipids than did those of control birds. The fact that the difference is increased to 3-fold when results are compared on a total organ basis implies that not only do the cells of the oestrogen-treated livers have an enhanced capacity to synthesize lipids from [1-^{14}C] acetate, but that there may be more cells present in the livers of the oestrogen-treated chicks. Once again, the results for the 30 - 31 h oestrogen-treated group were significantly greater than the control values at $12\frac{1}{4}$ - $14\frac{1}{4}$, 17 - $21\frac{3}{4}$ and $48\frac{3}{4}$ - $55\frac{1}{2}$ hours.

The results presented in Tables 6 and 7 show that expression of the incorporation data on a total liver basis or a cellular basis reveals or increases differences between values for oestrogen-treated and control chicks that are not obvious, or are smaller, when results are expressed per unit weight of liver. The values for the $48\frac{3}{4}$ - $55\frac{1}{2}$ h group are particularly interesting in this respect. Expression of these results as nmoles acetate incorporated/100 mg liver/hour revealed a mean value for the oestrogen-treated chicks which was slightly lower than the mean value for the control chicks, but the difference was not significant. On the other hand, when the results were expressed as nmoles acetate incorporated/liver/minute, the difference between the mean values was reversed. In other words, the mean value for the oestrogen-treated birds was greater than the mean value for the control birds, although the difference was not significant. These observations are perhaps not surprising, since 100 mg of an oestrogen-treated liver, at this time after injection, would be expected to contain fewer cells than 100 mg of a control liver and, therefore, the same numbers of cells are not being compared in the two cases. On the other hand, the livers of oestrogen-treated birds at this time are larger than control livers and possibly contain more cells and, therefore, the increased incorpor-

ation of [1-^{14}C] acetate by the livers of oestrogen-treated birds is not unexpected. Similar results were obtained for chicks that had received 6 injections prior to sacrifice (Table 7). Expression of the results on a cellular basis (nmoles acetate incorporated/0·1 mg DNA/h) revealed a significantly greater incorporation of [1-^{14}C] acetate into lipid by oestrogen-treated liver cells than by control liver cells at 48¾ - 55½ hours after a single injection (Table 6).

From the results presented in Figs. 13 - 15 and Table 6, it would appear that the greatest differences between values for oestrogen-treated and control birds were obtained at 17 - 21¾ hours after injection. Significantly greater [1-^{14}C] acetate incorporation values were obtained for oestrogen-treated chicks, compared with control chicks, at 17 - 21¾ hours after injection when results were expressed on a unit liver weight, a total liver weight, and a cellular basis, despite the fact that 100 mg of the oestrogen-treated livers probably contained fewer cells than 100 mg of the control livers. The results indicate that the oestrogen-treated cells have a far greater lipogenic capacity than the control cells, and that the increased total lipogenic capacity of the oestrogen-treated liver is primarily the result of this and, to a lesser extent, of a greater number of cells.

The results obtained for the distribution of radioactivity in the lipid classes separated by t.l.c., in the [1-^{14}C] acetate incorporation studies, showed that about 66·5 - 69·5% of the recovered radioisotope was in the triacylglycerol fraction, with phospholipids accounting for the next highest recovery of 13·0 - 16·4% (Table 9). These results are similar to those reported by Kudzma *et al.*[113], who also showed that [^{14}C] acetate was incorporated predominantly into triacylglycerol by chick liver slices, with phospholipid being the next important fraction. Kudzma *et al.*[113] treated 5-day old chicks with 0·1 mg diethylstil-

bestrol in sesame oil/day for 18 days and, after incubating liver slices with [^{14}C] acetate, reported that a greater proportion of the radioisotope was recovered in triacylglycerol and a lesser proportion in phospholipid for liver slices of the oestrogen-treated chicks (75% and 17% respectively) than for those of control chicks injected with sesame oil only (51% and 29% respectively). These results are in agreement with observations made in the present study. Similar distributions of ^{14}C-label from non-lipid precursors, such as acetate and glucose, in the lipid fractions of liver slices and hepatocytes from the domestic fowl, have been reported by Weiss et al. (237), Goodridge et al. (372) and Watkins et al. (375), and for the duck by Evans (24). The in vivo experiments performed by O'Hea & Leveille (21), to study lipogenesis in the chick, showed that after saponification, most of the ^{14}C-label from [1-^{14}C] acetate was recovered in fatty acid residues derived from complex lipids, as was found in the present study. These workers also showed similar distributions of the label in the triacylglycerol and phospholipid fractions as were found in the current study.

When the [^{14}C] acetate incorporation results were expressed /g wet weight of liver, Kudzma et al. (113) showed that livers from chicks treated with diethylstilbestrol (0·1 mg hormone each day for 18 days) incorporated 3-fold more acetate into total lipid than livers from control chicks. If the triacylglycerol fraction was considered alone, the difference was increased to 4·5-fold. Assuming that liver samples from the oestrogen-treated chicks contained fewer cells than samples from control chicks, and that oestrogen-treated livers were larger than control livers, the difference would probably be even greater if the results were expressed on a total liver or a cellular basis. The differences obtained in the present study (Fig. 13, Table 6) are not as obvious, which may have something to do with the use of 17β-oestradiol instead

of diethylstilbestrol, the duration and mode of oestrogen treatment, and the age and sex of the chicks. Nevertheless, it is of interest to note that after the publication of the data in Metab. Clin. Exp. in 1973, Kudzma and his co-workers made several attempts to repeat this work and failed to demonstrate increased lipogenesis by liver slices from oestrogen-treated chicks using [^{14}C] acetate (personal communication, 1980). Kudzma et al. (114) did, however, demonstrate that [1-^{14}C] acetate, administered in vivo to chicks, was incorporated to a greater extent into lipid by livers of chicks (850 - 1000 g body wt.) treated with a daily dose of diethylstilbestrol (2 mg/day) for 4 days than by livers of control chicks. These workers also measured the hepatic acetyl-CoA pool sizes for oestrogen-treated and control chicks, and found that the concentration of acetyl-CoA was the same in both cases. Therefore, on the basis of this result, it is probable that the differences in acetate incorporation between control and oestrogen-treated chicks observed in the present study and in the studies of Kudzma et al. (113, 114) actually represent differences in lipogenic capacity rather than differences in the intracellular pool size and specific radioactivity of the acetyl-CoA.

It is of interest at this point to note similar work that has been done using Xenopus laevis. Smith et al. (139) used [^{14}C] acetate to register a 120 to 160-fold increase in cholesterol and fatty acid synthesis in liver slices from female Xenopus laevis 6 days after they had been exposed to oestrogen treatment in vivo (1 mg 17 β-oestradiol/animal). Philipp & Shapiro (140) used [^{14}C] acetate to measure cholesterol and fatty acid synthesis in vitro in liver cubes from male Xenopus laevis at various times after the administration of 17 β-oestradiol in vivo (0·4 mg/100 g body wt.). These workers observed increased hepatic syntheses of cholesterol and fatty acids in these

animals compared with control animals, with peak values being obtained at 6 days after oestrogen treatment.

2. $^{3}H_2O$ incorporation studies

Since acetyl-CoA pool sizes were not determined in the present study, it is perhaps rather presumptuous to assume that the pool sizes determined by Kudzma et al. (114) can be adopted, particularly as the chicks were of different strains, age and perhaps sex, and were treated for different periods with different oestrogens. To minimize possible difficulties imposed by uncertainties about the pool size and specific radioactivity of acetyl-CoA leading to equivocal conclusions being drawn from the data, and to support the results obtained using [1-^{14}C] acetate, de novo lipogenesis by chick liver slices was also investigated using $^{3}H_2O$. The use of a ^{14}C-labelled substrate underestimates hepatic fatty acid synthesis, since it does not take into account acetyl-CoA formed from other substrates, whereas $^{3}H_2O$ is incorporated into fatty acids regardless of the nature of the acetyl-CoA precursor. It would, however, seem that the use of $^{3}H_2O$ to measure de novo lipogenesis in the livers of chicks after oestrogen treatment may not be ideal, since the increase in cell size (and cell water 'pool') caused by oestrogen treatment might be expected to lead to a decrease in the specific radioactivity of water in the cell and, therefore, to an underestimate of hydrogen incorporation into lipid.

De novo lipogenesis by livers of untreated, control and oestrogen-treated chicks was studied by incubating liver slices in avian bicarbonate buffer, pH 7·4, containing $^{3}H_2O$, for 1 hour at 40°C. Adoption of an incubation period of 1 hour was based on the data presented in Fig. 16, and the initial lag period was undoubtedly a result of the delay in the diffusion of $^{3}H_2O$ into the cells and equilibration with the unlabelled

cellular water, and of the time required for thermoequilibration of the tissue in the incubation medium. The reasons for using an incubation temperature of 40°C have been explained previously. The results of the $^{3}H_2O$ incorporation studies were presented as µg atoms H incorporated into lipid/100 mg liver/hour, as µg atoms H incorporated into lipid/liver/minute and as µg atoms H incorporated into lipid/0·1 mg liver DNA/hour (Figs. 17 - 19, Table 10), for reasons explained earlier for the [1-^{14}C] acetate incorporation studies.

When the rate of incorporation of $^{3}H_2O$ into total lipid was expressed on the basis of a unit wet weight of liver (µg atoms H incorporated/100 mg liver/h), it became clear that none of the results for the oestrogen-treated groups differed significantly from the results for their corresponding control groups, although the mean value for the oestrogen-treated chicks was greater than the mean value for the control chicks in each post-injection time group. This picture of the data was altered, however, when the results were expressed on the basis of total hepatic lipogenic capacity (µg atoms H incorporated/liver/min) as shown in Fig. 18 and Table 10. In this case, the values for the oestrogen-treated birds in the 14 - 17 h, 19 - 21¾h, 25¼ - 28¾ h and 40¼ - 43¼ h groups were significantly greater than their corresponding control values. Insufficient data prevented statistical analysis of the results in the 29¼ - 31½ h group. These results reflect those obtained in the [1-^{14}C] acetate incorporation studies, since, although the lipogenic capacity of a unit weight of oestrogen-treated liver was not significantly greater than that of control liver in the time groups mentioned above, the total lipogenic capacities of the oestrogen-treated livers were significantly greater by virtue of an increase in liver size. Expression of the $^{3}H_2O$ incorporation data on a cellular basis (µg atoms H incorporated/0·1 mg DNA/h) also revealed significantly

greater values for oestrogen-treated chicks than for control chicks in the 14 - 17 h, 19 - 21¾ h, 25¼ - 28¾ h and 40¼ - 43¼ h post-injection time groups. Therefore, in these time groups, the oestrogen-treated cells had a greater lipogenic capacity than the control cells. Comparison of the $^{3}H_2O$ incorporation data expressed as the total organ lipogenic capacity with that expressed on a cellular basis (Table 10) suggests that the oestrogen-dependent stimulation of lipogenesis is mainly caused by an enhanced rate of lipogenesis per cell and, to a lesser extent, by an increase in cell number. Significant differences were not obtained for the chicks in the 3½ - 7¼ h group, regardless of how the data were expressed. An interesting observation resulting from these experiments was that, in whatever way the results were presented, the values for the untreated chicks were significantly lower than the results for the control chicks in the 3½ - 7¼ h and 14 - 17 h groups, suggesting that fatty acid synthesis may be stimulated at these times by handling and/or the injection of propane-1,2-diol. From Figs. 17 - 19 and Table 10, it would appear that the greatest differences in $^{3}H_2O$ incorporation into lipid between oestrogen-treated and control birds were in the 40¼ - 43¼ h group, although the differences appear to be, at least partly, the result of low control values. The distribution of the ^{3}H-label in the lipid classes separated by t.l.c. was similar to that obtained in the [1-^{14}C] acetate studies, and to the results obtained by other workers which have been mentioned previously. However, in these $^{3}H_2O$ experiments, no significant differences were obtained between control and oestrogen-treated chicks in the distribution of the radioisotope in the lipid classes.

In contrast to the many studies that have been carried out using ^{14}C-labelled non-lipid precursors of fatty acids to study hepatic lipogenesis in oestrogen-treated and control chicks, the use of $^{3}H_2O$ has

been rare. Dashti *et al.* [365] used ^3H_2O to measure hepatic lipogenesis *in vivo* in 19-day old male turkeys treated with diethylstilbestrol, and reported a 3·4-fold increase in the incorporation of tritium from ^3H_2O into liver triacylglycerol when compared with control birds. In a later report, these workers treated 19-day old male turkeys with a single dose of diethylstilbestrol (40 mg/kg body wt.), and injected birds with ^3H_2O 1 hour prior to sacrifice [252]. Total hepatic triacylglycerol synthesis, 24 - 48 hours after oestrogen treatment, was found to be increased 8 to 12-fold compared with control values.

3. [9,10-^{3}H] Palmitate incorporation studies

Since the results of the [1-^{14}C] acetate and ^3H_2O studies did not reveal dramatic differences in the capacities of oestrogen-treated and control chick livers for *de novo* lipogenesis *in vitro*, a final study was performed using [9,10-^{3}H] palmitate to measure the capacities of the livers for incorporating pre-formed fatty acids into complex lipids *in vitro*. The results of the previous experiments employing [1-^{14}C] acetate and ^3H_2O, and those of a number of other workers [21, 113, 237, 372, 375], have shown that triacylglycerols are the major product of lipogenesis in chick liver and, therefore, the incorporation of [9,10-^{3}H] palmitate into triacylglycerol by chick liver slices was measured.

Liver slices from untreated, control and oestrogen-treated chicks were incubated in avian bicarbonate buffer, pH 7·4, for 1 hour at 40°C, in the presence of 12 mM-β-D(-)-fructose and 0·65 mM-[9,10-^{3}H] palmitate. The incubation period of 1 hour was adopted after obtaining the data presented in Fig. 20, and the incubation temperature was 40°C as used previously. The lack of an initial lag phase implies that the labelled fatty acid enters the cell rapidly and that the intracellular pool size

must be very small, or have a quick turnover, to enable rapid equilibration of the labelled palmitate with unlabelled fatty acids. The concentration of palmitate employed (0·65 mM) was adopted after obtaining the data presented in Fig. 21 and for the reasons outlined in the Results section. Hawkins & Heald [236] used a similar palmitate concentration, and the inclusion of 12 mM-β-D(-)-fructose in incubations was adopted from their studies, since they showed that this enhanced the incorporation of 0·62 mM-[1-^{14}C] palmitate into the neutral lipid fractions of liver slices from the domestic fowl.

Results of studies on the incorporation of palmitate into triacylglycerol were presented (Figs. 22 - 24, Table 11) on the basis of a unit wet weight of liver (nmoles incorporated/100 mg liver/h), as the total hepatic capacity (nmoles incorporated/liver/min) and on a cellular basis (nmoles incorporated/0·1 mg liver DNA/h). Hence, any differences that might exist in the capacities of the livers to incorporate [9,10-^{3}H] palmitate into triacylglycerol, either at the cellular level or on the level of the whole organ, would become apparent.

When the data were presented on the basis of a unit wet weight of liver (nmoles palmitate incorporated/100 mg liver/h), it was found that the results for oestrogen-treated chicks were significantly greater than corresponding control values in the 19 - 22¼ h, 28 - 31¾ h and 40½ - 44 h groups. In these groups, the mean incorporation values for hormone-treated birds were 1·19-fold, 1·27-fold and 1·29-fold higher, respectively, than the corresponding control values. Expression of the results as total hepatic capacity (nmoles palmitate incorporated/liver/min) revealed significant differences between oestrogen-treated and control chicks at 14½ - 18½, 19 - 22¼, 28 - 31¾ and 40½ - 44 hours, when the mean incorporation values for the hormone-treated chicks were 1·61-fold, 1·62-fold, 2·26-fold and 2·47-fold higher, respectively, than the

corresponding control values. Thus, expression of the results as total hepatic capacity increased the differences between oestrogen-treated and control birds, indicating that the increased capacity to incorporate palmitate into triacylglycerol was, at least in part, the result of an increase in liver size in the oestrogen-treated chick. Oestrogen-treated birds in the $3\frac{3}{4}$ - 8 h and $45\frac{1}{2}$ - $51\frac{3}{4}$ h groups showed no significant differences in palmitate incorporation compared with corresponding control birds when the results were expressed either on the basis of a unit wet weight of liver or as total hepatic capacity, although the mean value for the total hepatic capacity of the $45\frac{1}{2}$ - $51\frac{3}{4}$ h oestrogen-treated group was 1·61-fold greater than that of the corresponding control group.

In Fig. 24 and Table 11, the results were expressed on a cellular basis, and the values for the oestrogen-treated chicks in the $14\frac{1}{2}$ - $18\frac{1}{2}$ h, 19 - $22\frac{1}{4}$ h, 28 - $31\frac{3}{4}$ h and $40\frac{1}{2}$ - 44 h groups were significantly greater than the corresponding control values, although the differences between the oestrogen-treated and control values appear to be, at least partly, due to low control values. The mean incorporation values for these groups of hormone-treated chicks were 1·35-fold, 1·54-fold, 1·72-fold and 1·87-fold higher, respectively, than the corresponding control values. The DNA contents of the livers in the $45\frac{1}{2}$ - $51\frac{3}{4}$ h group were not determined, but the results expressed on a cellular basis for the oestrogen-treated chicks in the $3\frac{3}{4}$ - 8 h group were not significantly greater than the corresponding control values. Consequently, in the $14\frac{1}{2}$ - $18\frac{1}{2}$ h, 19 - $22\frac{1}{4}$ h, 28 - $31\frac{3}{4}$ h and $40\frac{1}{2}$ - 44 h groups, the cells of the oestrogen-treated livers had a greater capacity than those of the control livers for incorporating palmitate into triacylglycerol. In whatever way the results were expressed, the values for the untreated chicks were not significantly different from any of the control values.

This would indicate, therefore, that the incorporation of fatty acid into triacylglycerol is not stimulated by handling and/or the injection of propane-1,2-diol. In contrast, the $^{3}H_2O$ incorporation studies indicated that fatty acid synthesis was stimulated at early times ($\leq$ 17 h) after the injection of propane-1,2-diol.

Hawkins & Heald [236] demonstrated that liver slices from laying hens and from oestrogen-treated 11-week old female chickens (2 mg oestradiol benzoate/bird on alternate days for 7 days) incorporated more palmitate into neutral lipids than did liver slices from untreated immature female birds when results were expressed on both a dry weight basis and a cellular basis. These workers also measured the hepatic free fatty acid pool sizes for immature female birds and laying hens, and found a greater pool size in the laying birds. In connection with this, the specific radioactivity of the liver free fatty acid was lower in the laying birds than in the immature birds. Therefore, the greater incorporation of palmitate into neutral lipids by the laying birds was 'real', and not due to a smaller free fatty acid pool and a higher specific radioactivity. In addition, Balnave [164] observed higher liver free fatty acid levels for oestrogen-treated immature pullets than for control birds. The free fatty acid pool sizes of the livers were not measured in the present study, and so the results obtained cannot be considered to be the true relative rates of triacylglycerol synthesis from palmitate for the livers of untreated, control and oestrogen-treated chicks. However, it would seem likely that oestrogen treatment of the male chick would cause similar changes to those observed after oestrogen treatment of the immature pullet, and in the mature hen in response to endogenous oestrogen. Consequently, it might be expected that the hepatic fatty acid pool size would be larger, and the specific radioactivity of the fatty acid lower, for oestrogenized male chicks than for control and untreated male chicks.

In the [1-^{14}C] acetate studies, the greatest differences in incorporation, on a total organ basis and a cellular basis, between oestrogen-treated and control male chicks were in the 17 - 21$\frac{3}{4}$ h group. In the $^{3}H_2O$ and [9,10-^{3}H] palmitate studies, significant differences became apparent at, or after, 14 and 14$\frac{1}{2}$ hours post-injection, respectively, with the greatest differences being observed at about 40 - 44 hours, although some of this difference appeared to be due to a decrease in control values. In none of the studies were significant differences observed between oestrogen-treated and control chicks from 3 to 8 hours after injection, and significant differences only became evident at, or after, 14 hours post-injection. The results of these experiments demonstrated that the livers of oestrogenized male chicks developed enhanced capacities to incorporate non-lipid precursors into fatty acids and to incorporate fatty acids into triacylglycerol. However, the extent to which the fatty acids that are incorporated into triacylglycerol are of hepatic origin or are derived from other sources, such as adipose tissue, cannot be deduced from the results of the present study. The possible importance of extra-hepatic sources of fatty acids cannot be ruled out, particularly since it is suggested that such sites may contribute up to 50% of the total fatty acids synthesized by the chick [3, 4]. Nevertheless, this lack of knowledge does not detract from the conclusions drawn from the incorporation studies, that the livers of oestrogenized chicks develop an increased capacity to produce triacylglycerol from both non-lipid precursors and free fatty acids.

CHAPTER 4

THE EFFECT OF 17β-OESTRADIOL INJECTION ON HEPATIC FATTY ACID SYNTHETASE ACTIVITY OF THE MALE CHICK

INTRODUCTION

1. De novo lipogenesis and associated lipogenic enzymes

The rate of lipogenesis and the activities of several lipogenic enzymes have been extensively studied in a variety of animals during development and subsequent maturity. In many cases, changes in the nutritional or hormonal status of the animal promote changes in the rate of lipogenesis, often accompanied by corresponding changes in the activities of key lipogenic enzymes, especially in the liver (6, 44, 393 - 396). The most popular experimental animals for this work have been mammals where, usually, the liver and adipose tissue are the major sites of lipogenesis. However, birds are particularly useful experimental animals for the study of hepatic lipogenic enzymes, since the liver is the major site of de novo lipogenesis in avian species (21, 23 - 27, 34).

The de novo biosynthesis of long-chain saturated fatty acids from acetyl-CoA is catalysed by the two cytosolic enzyme systems, acetyl-CoA carboxylase (EC 6.4.1.2) and fatty acid synthetase (393, 396). These two enzyme systems catalyse the following sequence of reactions:-

$$\text{Acetyl-CoA} + \text{ATP} + HCO_3^- \xrightarrow[\text{carboxylase}]{\text{acetyl-CoA}} \text{malonyl-CoA} + \text{ADP} + P_i \qquad \text{(a)}$$

$$\text{Acetyl-CoA} + 7\ \text{malonyl-CoA} + 14\ \text{NADPH} + 14\ H^+ \xrightarrow[\text{synthetase}]{\text{fatty acid}}$$

$$\underset{(C_{16:0})}{\text{palmitate}} + 14\ NADP^+ + 7\ CO_2 + 8\ \text{CoASH} + 6\ H_2O \qquad \text{(b)}$$

The formation of malonyl-CoA from acetyl-CoA is the first committed step in the fatty acid synthetic sequence, and is regarded as the rate-

limiting step in de novo fatty acid synthesis for a number of tissues (19, 397 - 402). In short-term regulation of fatty acid synthesis, the activity of a constant quantity of acetyl-CoA carboxylase is postulated to be controlled by the phosphorylation state of the enzyme (403, 404), the concentrations of allosteric effectors such as citrate and long-chain acyl-CoA derivatives (405, 406), and the availability of substrates for lipogenesis (375, 394, 400, 404, 407). However, long-term regulation has been found to involve changes in the concentrations of acetyl-CoA carboxylase and other lipogenic enzymes, brought about by alterations in their rates of synthesis and degradation (408 - 411).

The NADPH required for the reductive stages of fatty acid synthesis is usually provided by the action of 'malic' enzyme (EC 1.1.1.40) and/or the pentose phosphate pathway dehydrogenases, glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) as follows:-

$$\text{L-Malate} + \text{NADP}^+ \xrightarrow[\text{enzyme}]{\text{'malic'}} \text{pyruvate} + CO_2 + \text{NADPH} + H^+ \qquad \text{(c)}$$

$$\alpha\text{-D-Glucose 6-phosphate} + \text{NADP}^+ \xrightarrow[\text{dehydrogenase}]{\text{glucose 6-phosphate}} \text{6-phosphogluconolactone} + \text{NADPH} + H^+ \qquad \text{(d)}$$

$$\text{6-Phosphogluconate} + \text{NADP}^+ \xrightarrow[\text{dehydrogenase}]{\text{6-phosphogluconate}} \text{ribulose 5-phosphate} + CO_2 + \text{NADPH} + H^+ \qquad \text{(e)}$$

Several workers have suggested that the pentose phosphate pathway is of little importance in providing reducing power for fatty acid synthesis in birds (32, 49, 269, 412 - 414). The pathway has been shown to be active in the chick embryo, but its activity declines as incubation progresses, until at hatching it is virtually inactive as in the

adult bird [415]. In contrast, the pentose phosphate pathway is an important source of NADPH for the reductive reactions of lipogenesis in mammals [393, 416].

The acetyl-CoA required for reaction (a) may be provided by the cleavage of citrate in the cytoplasm, catalysed by ATP citrate lyase (EC 4.1.3.8):-

$$\text{Citrate} + \text{ATP} + \text{CoASH} \xrightarrow[\text{lyase}]{\text{ATP citrate}} \text{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + P_i \qquad \text{(f)}$$

The saturated long-chain fatty acids produced as a result of reactions (a) and (b) may be converted to unsaturated fatty acids by the action of desaturation enzyme systems [417]. Fatty acids may be incorporated into complex lipids by the pathways shown in Fig. 10.

2. The effects of the pattern of food intake and the composition of the diet on hepatic lipogenesis and lipogenic enzyme activities

The rate of hepatic lipogenesis is closely related to nutritional state, and to the composition of the diet given to an animal [6, 8, 44, 47, 393]. For example, starvation or feeding a high fat diet have been shown to be accompanied by depressed hepatic lipogenesis and corresponding decreases in lipogenic enzyme activities in several animal species. Conversely, refeeding after starvation or feeding a low fat, high carbohydrate diet are accompanied by increased hepatic lipogenesis and increases in lipogenic enzyme activities. Such effects are observed in birds [1, 32, 45, 48 - 50, 237, 410, 418 - 422]. In the rat, refeeding after starvation produces elevated liver lipogenesis and lipogenic enzyme activities to levels far greater than in *ad libitum*-fed animals [1, 6, 47]. Under similar circumstances, increased hepatic lipogenesis is observed in birds [45, 46], but the enormous 'overshoot' in lipogenic

enzyme activities is not found, and recovery is to normal or slightly elevated levels (1, 32, 45, 46, 48 - 51). This may have something to do with the higher lipogenic enzyme activities in avian liver, and implies that lipogenesis may be more strictly regulated in bird liver than in rat liver.

3. Changes in hepatic lipogenesis and lipogenic enzyme activities during development

The development of birds and mammals is accompanied by major changes in nutritional and hormonal status, which promote changes in hepatic lipogenesis (423). Developmental studies in birds have been facilitated by the accessibility and independence of the developing avian embryo (351, 372, 413, 424 - 426). In chicks, hatching results in a dietary change from a high fat, low carbohydrate food source (egg yolk) to a low fat, high carbohydrate food intake (cereal-based mash), and is accompanied by a large increase in hepatic lipogenesis (30) and the activities of certain lipogenic enzymes. Under these circumstances, increases in the activities of acetyl-CoA carboxylase, fatty acid synthetase, ATP citrate lyase and 'malic' enzyme have been demonstrated (413, 424, 427 - 430). It is generally considered that enzyme activity changes during development are the result of protein synthesis rather than the activation of latent proteins (410, 422, 424). However, some evidence has been presented which suggests the presence of some pre-existing inactive enzyme proteins in embryonic and neonatal chick liver (381, 431, 432).

That the emergence of enzyme activity on hatching is not prompted solely by the dietary change is shown by the fact that significant levels of certain hepatic lipogenic enzymes appear even if the newly hatched chicks are not fed. Increases in the activities of acetyl-CoA carboxylase, fatty acid synthetase and ATP citrate lyase occur during

the hatching period before feeding begins, but an increase in 'malic' enzyme activity is not seen at this time [413, 424, 429, 433]. After hatching, hepatic ATP citrate lyase and fatty acid synthetase activities continue to increase when the chicks are not fed, but the activities of 'malic' enzyme and acetyl-CoA carboxylase do not change. On feeding, 'malic' enzyme and acetyl-CoA carboxylase activities increase, and the activities of ATP citrate lyase and fatty acid synthetase increase at a much faster rate than in the absence of food intake [49, 424, 433]. Some of the effects of feeding a mash diet have been shown to be mimicked in unfed neonatal chicks by the injection of glucose or fructose, or by glucose feeding [351, 381, 410, 424, 432, 434]. Some controversy exists as to whether glucose or fructose injection evokes similar effects in chick embryo liver [351, 424, 435]. Goodridge [424] has shown that hepatic fatty acid synthetase activity can be increased by exposing chick embryos to an atmosphere of 100% oxygen for 24 hours, implying that environmental factors other than diet may be involved in lipogenic enzyme development.

In contrast, in the rat, birth results in a dietary change from a low fat, high carbohydrate nutrient supply provided via the placental-maternal circulation, to a high fat, low carbohydrate supply of milk. This dietary change is accompanied by a substantial reduction in hepatic lipogenesis and the activities of certain lipogenic enzymes [436, 437]. Later, weaning represents a second dietary transition, from the high fat, low carbohydrate maternal milk to a low fat, high carbohydrate cereal-based feed, and this is accompanied by an increase in hepatic lipogenesis and the activities of key lipogenic enzymes [436, 437].

4. The effects of hormones on hepatic lipogenesis and lipogenic enzyme activities

(a) Effects of insulin, glucagon, catecholamines, thyroid hormones and prolactin

Nutritional changes, such as those described above, often promote changes in the levels of certain hormones, notably insulin and glucagon, whose actions at the cellular level provide a molecular explanation for the observed changes in lipogenesis and lipogenic enzyme activity. Birds and mammals differ considerably in the endocrine control of carbohydrate and lipid metabolism (416, 438, 439). Insulin inhibits lipolysis in rat adipose tissue (440), but in the chicken insulin is not anti-lipolytic (441), and the plasma free fatty acid level increases in response to this hormone (441, 442). In addition, unlike rat adipose tissue, avian adipose tissue responds poorly, if at all, to the lipogenic action of insulin (32, 443, 444). In mammals, hepatic lipogenesis is stimulated by insulin, and, in the chicken, hepatic lipogenesis (276) and lipogenic enzyme activities (444) have been stimulated using high doses of insulin. Glucagon appears to be the major lipolytic hormone in birds (441, 445 - 448), and fatty acid synthesis in cultured chicken hepatocytes has been shown to be inhibited by glucagon (375). In birds, insulin potentiates the stimulation of lipolysis brought about by glucagon (449), whereas, in rat adipose tissue, insulin is antagonistic to glucagon-stimulated lipolysis (440).

Catecholamines are activators of lipolysis in mammalian adipose tissue (450), but have been shown to be relatively insignificant in fatty acid mobilization in birds (445, 449). The comparative lack of response of avian adipose tissue to insulin and catecholamines may be associated with the minor role that adipose tissue plays in lipogenesis in these species. Thyroid hormones stimulate fatty acid synthesis and lipogenic enzyme activities in animals that have free access to food and

water [451 - 454]. The action of thyroxine on lipogenic enzyme activity has been studied in chickens, with the conclusion that thyroxine increases basal metabolism and, if food and water are restricted, reduces the energy available for fatty acid synthesis. When food and water were supplied ad libitum, hypophysectomy was shown to depress hepatic 'malic' enzyme activity, and thyroxine injection to increase hepatic 'malic' enzyme activity in intact and hypophysectomized birds [455]. If birds were maintained on equalized food and water intakes, thyroxine reduced rather than increased hepatic 'malic' enzyme activity in normal male chicks [456].

The emergence of hepatic lipogenic enzyme activities during the development of birds has been extensively studied with respect to possible hormonal influences. Thyroid hormones have been shown to increase the activities of acetyl-CoA carboxylase [433], ATP citrate lyase [372], fatty acid synthetase [372, 433] and 'malic' enzyme [372, 374] in cultured chick embryo hepatocytes. After the in vivo administration of insulin, glucagon, and cyclic AMP, insulin was implicated as being the active hormone in stimulating chick embryo hepatic fatty acid synthetase activity [426]. The in vitro treatment of chick embryo hepatocytes and liver explants with insulin alone, or in combination with thyroid hormones, has been reported to stimulate acetyl-CoA carboxylase, fatty acid synthetase and 'malic' enzyme activities [374, 433, 457]. Contradictory observations have been reported as regards glucagon, since Ryder & Campos [458] found that this hormone stimulated acetyl-CoA carboxylase activity in chick embryo hepatocytes, whilst other workers have reported inhibition of lipogenesis and lipogenic enzyme activities [372, 374]. It is apparent, therefore, that studies on the role of hormonal influences in the emergence of lipogenic enzyme activities during avian development are both incomplete and controversial.

Prolactin has been implicated to play an important role in the stimulation of hepatic lipogenesis and fat storage in migratory species (459, 460). Goodridge & Ball (268, 269) studied the effects of prolactin and growth hormone on lipogenesis, and showed that prolactin was responsible for the induction of a food-dependent increase in hepatic lipogenesis and associated enzyme activities in the pigeon. Similar metabolic changes were observed with growth hormone, although the increase in size of the crop sac, seen with prolactin, was not obtained.

(b) Effects of gonadal hormones

The effects of gonadal hormones on avian lipid metabolism have been widely studied, with special emphasis being placed on oestrogens, which have major involvement in the physiological changes observed when the hen comes into lay (82). However, androgens and progestagens have been shown to be involved in these physiological changes (166), and androgens exert significant influences on hepatic lipid metabolism and lipogenic enzyme activities in chickens (165, 166, 245, 253, 295, 363, 461). For example, testosterone treatment of 4-week old pullets (2 mg testosterone propionate/bird) has resulted in increases in the specific activities of hepatic ATP citrate lyase and 'malic' enzyme after 1 day of hormone treatment, but after longer periods, with hormone administration on alternate days, variable effects were obtained (166, 363). Pearce (253) injected laying hens with a daily dose of testosterone propionate (1 mg/kg body wt.) for 4 days and, 24 hours after the last injection, observed significantly reduced specific activities of hepatic ATP citrate lyase and 'malic' enzyme in these birds. These reports clearly indicate the importance of considering age of animal, hormone dose level and duration of treatment when attempting to elucidate hormonal effects on metabolism. In addition, testosterone treatment significantly affects liver lipid

metabolism by increasing the oxidation of lipids [245, 461], an effect not found with oestrogen treatment. The involvement of progestagens in the regulation of lipogenesis is unclear, as administration of progesterone has not been found to have marked effects on hepatic lipid metabolism or enzyme activities in the domestic fowl [165, 166, 295].

Studies on the development of sexual maturity in the domestic fowl have shown that the laying hen has greater hepatic specific activities of ATP citrate lyase and 'malic' enzyme than does the non-laying hen or cockerel, but has similar specific activities to those found in young birds aged 4 - 7 weeks [125, 246, 378]. These observations are in agreement with the work of Leveille [239], who found similar levels of hepatic lipogenesis in the laying hen and the young bird.

The administration of oestrogen to immature female birds results in biochemical and physiological changes similar to those found when the female comes into lay [82]. For example, short-term oestrogen treatment (≤ 2 days, 1 - 2 mg oestradiol dipropionate / bird) leads to increased specific activities of ATP citrate lyase and 'malic' enzyme in the livers of 4-week old pullets [126, 166, 256, 363]. After prolonged oestrogen treatment (4 mg β-oestradiol-3-benzoate/bird/day for 6 days), increased total and specific activities of fatty acid synthetase have been demonstrated in the oviduct and liver of 1-month old pullets [31]. Pageaux *et al.* [235] have reported a rapid increase in the specific activity of hepatic acetyl-CoA carboxylase in 16-day old female quail following a single injection of oestradiol benzoate (0·2 mg/kg body wt.), with peak activity being recorded at 3 hours after injection. However, the lack of control values at the varying times after injection does not allow conclusive interpretation of the data. It is possible that the increased acetyl-CoA carboxylase activity registered at 3 hours was a stress-evoked response. It could be argued that stress would not be

expected to lead to an increase in lipogenic enzyme activity but rather, if any, to an increase in lipolytic enzyme activity. However, if stress due to handling and/or injection resulted in hyperphagia, then increased lipogenic enzyme activities might be expected [270]. This study clearly shows the need for sham-injected control animals at all stages of experimental work, since untreated animals do not provide a valid comparison. As regards mammals, oestrogen treatment of female rats has been shown to cause increased uterine activities of fatty acid synthetase, ATP citrate lyase, 'malic' enzyme and other related enzymes [285], and also increased hepatic activities of fatty acid synthetase, acetyl-CoA carboxylase and 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34) [366, 462].

It has been demonstrated that oestrogen treatment of male birds and amphibia initiates changes in liver metabolism, including vitellogenesis and enhanced lipogenesis, similar to those found when the female comes into lay [63, 81, 115, 141, 265]. It is reasonable to believe that oestrogen treatment of male birds, resulting in fatty liver and lipaemia, involves hepatic lipogenic enzyme activity changes similar to those observed as the hen approaches lay. In this situation, it is of interest to determine whether the concentrations of lipogenic enzymes are increased, and if so, which enzymes are affected, and to elucidate the time courses and functional significance of such changes.

An increase in the specific activity of 'malic' enzyme was noted by de Vellis & Schjeide [244] for the livers of oestrogenized roosters. Lippiello *et al.* [296] obtained increased stearyl-CoA desaturase (EC 1.14.99.5) specific activity in rooster liver, up to 48 hours after birds had received a single injection of 17β-oestradiol (3·5 mg/100 g body wt.). After treating 1-week old male chicks with a daily injection of 1 mg diethylstilbestrol for 1 - 3 days, and killing the birds 4 hours after the last injection in each case, the specific activity of

hepatic choline kinase (EC 2.7.1.32) was found to have increased 1·7-fold at 4 hours after one injection of diethylstilbestrol, and this became a 3-fold increase after 3 injections. In addition, the specific activity of hepatic phosphatidylethanolamine-N-methyltransferase (EC 2.1.1.17) increased nearly 2-fold after 2 injections, and this increase was maintained after 3 injections [463, 464]. Treatment of male and female 6 to 11-day old chicks with daily injections of 2 mg diethylstilbestrol over 5 days resulted in increased total liver activities of fatty acid CoA ligase (AMP-forming) (EC 6.2.1.3), sn-glycerol 3-phosphate acyl-CoA acyltransferase (EC 2.3.1.15) and diacylglycerol acyltransferase (EC 2.3.1.20), without changes in their specific activities [254]. The total hepatic activity of diacylglycerol cholinephosphotransferase (EC 2.7.8.2) was found to increase, with a concomitant decrease in specific activity.

The reaction catalysed by acetyl-CoA carboxylase has been implicated by Philipp & Shapiro [140] as the regulatory step in hepatic fatty acid synthesis following oestrogen treatment of male Xenopus laevis. These workers showed that the administration of 17β-oestradiol in vivo resulted in increased hepatic fatty acid and cholesterol synthesis with concurrent increases in acetyl-CoA carboxylase and 3-hydroxy-3-methylglutaryl-CoA reductase activities, whilst fatty acid synthetase activity remained unchanged. Once again, control values were not presented, so it is not possible to make conclusive deductions about the action of the hormone from the data supplied.

An interesting point arising from the gonadal hormone studies is that, although lipogenic enzyme activities may be increased at early times after hormone treatment, prolonged treatment and/or high doses often result in normal or depressed levels of activity [166, 246, 253, 256]. It would appear that short-term hormone treatment ($\leq$ 2 days), at

a carefully selected dose, is more indicative of physiological effects than long-term administration and/or high dosage when pharmacological effects might predominate [256].

Some of the enzyme studies carried out on birds have demonstrated interesting contradictions to the observations of coordinate changes in the rate of lipogenesis and lipogenic enzyme activities which occur under many circumstances [395]. Most notable are the reports of changes in lipogenic enzyme activities that are not accompanied by changes in the rate of lipogenesis [49, 351, 424], changes in the rate of lipogenesis that occur without, or before, changes in lipogenic enzyme activities [33, 45, 46, 375, 410, 420, 424], changes in the rate of lipogenesis and enzyme activity which are similar in direction but very different in magnitude [46, 49, 50], and non-coordinate changes in lipogenic enzyme activities [49, 424]. These findings infer that increases in the concentrations of lipogenic enzymes may not always be responsible for increased rates of lipogenesis, and that changes in lipogenesis may involve an altered flux of metabolites through the biochemical pathways and the regulation of the activities of constant amounts of enzymes [276, 393, 465].

The catalytic capacities of the fully activated acetyl-CoA carboxylase and fatty acid synthetase have been shown to be similar in some animal tissues [395, 466], but it is suggested that, <u>in vivo</u>, the carboxylase is inhibited to a variable extent and does not exhibit maximum catalytic efficiency [44, 375, 394, 395, 467, 468]. Apart from the obvious limit imposed by substrate availability, acetyl-CoA carboxylase activity is controlled by covalent and allosteric modifications of the enzyme [407, 469]. Acetyl-CoA carboxylase exists in a depolymerized inactive state, and a polymerized active form [470, 471]. The interconversion of these states can be effected by phosphorylation and

dephosphorylation of the enzyme, the phosphorylated form being less active, largely because of the reduced affinity of the enzyme for the allosteric activator citrate (403, 404, 472 - 478). The allosteric regulation of acetyl-CoA carboxylase is also considered to operate through changes in the aggregation state of the enzyme. Therefore, citrate and isocitrate enhance polymerization with an increase in enzyme activity (406, 479, 480), while long-chain acyl-CoA esters encourage depolymerization and reduced activity (396, 468, 469, 471, 481 - 483). In addition, physiological concentrations of CoA have been shown to activate acetyl-CoA carboxylase (484, 485). Fatty acid synthetase has also been found to exist in a phosphorylated form with reduced activity, and a dephosphorylated active form (486). Fatty acid synthetase is activated by various phosphorylated sugars (487, 488), and inhibited by fatty acyl-CoA derivatives (489 - 492) and high concentrations of CoA (493). In addition, both acetyl-CoA carboxylase and fatty acid synthetase are inhibited by high concentrations of malonyl-CoA (395, 466, 488, 494, 495). It is, therefore, possible that *in vivo*, in addition to alterations in the rates of synthesis and degradation of fatty acid synthetase, covalent modulators and allosteric effectors may significantly alter the activity of fatty acid synthetase. It has been suggested that acetyl-CoA carboxylase may not be the rate-limiting enzyme in fatty acid synthesis under all conditions (401, 466, 496). The possibility of chicken liver fatty acid synthetase exerting a regulatory effect on the rate of *de novo* fatty acid synthesis, at early times after oestrogen treatment, has not been excluded.

The aim of the present study was to investigate the effect of oestrogenization on hepatic fatty acid synthetase activity in the male chick. Optimum conditions for assay of the enzyme were determined, and used to study the effect of varying 17β-oestradiol dose level and varying time after hormone injection on enzyme activity.

METHODS

1. Preparation and assay of acetyl-CoA solutions

Acetyl-CoA was prepared by the method of Smith et al. [399], in which acetic anhydride was reacted with CoA in bicarbonate solution. The method of Chase [497] was used to determine the concentration of acetyl-CoA. In this assay, given an excess of oxaloacetate and DTNB, the difference between the free thiol content of the preparation and the total thiol groups resulting after incubation with citrate synthase was used as an estimate of acetyl-CoA concentration. The release of 5-thio-nitrobenzoate was registered by an increase in absorbance at 412 nm. Acetyl-CoA solutions were diluted to the required concentration (0·5 mM) with distilled water, and were stored at -20°C at pH 1 - 3. Fresh acetyl-CoA solutions were prepared every fortnight.

2. Preparation and storage of other solutions

Potassium phosphate buffers (1 M) of a variety of pH values (6·2 - 8·0), and 100 mM-EDTA solutions adjusted to pH 7·0 with KOH, were stored at 4°C and replaced at weekly intervals.

DTT (100 mM) and malonyl-CoA (1 mM) solutions were stored at -20°C, and fresh solutions were made fortnightly.

NADPH solutions (4 mM) were made just before use. The concentration of NADPH solutions was determined spectrophotometrically at 340 nm, given that an absorbance of 0·622 corresponds to a concentration of 100 nmoles NADPH/ml, using a 1 cm light path.

Homogenization buffer, comprising 0·2 M-potassium phosphate buffer (pH 7·0) and 1 mM-DTT, was prepared fresh each day. An assay 'cocktail', containing 0·25 M-potassium phosphate buffer (pH 6·2 - 8·0), 1·25 mM-DTT and 1·25 mM-EDTA (pH 7·0), was prepared just before use.

3. Protein determination

The protein concentration of each enzyme preparation was determined by the biuret method as described by Hübscher et al. [498], with some modification. The biuret reagent of Weichselbaum [499] was modified in the following way. Biuret reagent (200 ml) and 1 M-NaOH (160 ml) were mixed. Potassium iodide (4 g) was added and dissolved, and the solution was made up to 1 litre with distilled water. The resulting solution was stored at 4°C.

Duplicate protein samples were precipitated with an equal volume of ice-cold 10% (w/v) TCA, and the precipitate was collected by centrifugation at 4°C. The supernatant was decanted, and the pellet was dissolved in 1 ml 0·1 M-NaOH. The modified biuret reagent (2 ml) was added and colour, as measured by absorbance at 555 nm, was developed by incubation at 37°C for 15 minutes. Bovine serum albumin (fatty acid poor) was used as the standard over the protein range 0 - 10 mg.

4. Procedure for enzyme preparation

Since fatty acid synthetase is a soluble cytoplasmic enzyme [496, 500], a particle-free supernatant (PFS) was prepared from each liver under investigation. All operations were performed at 0 - 4°C.

Birds were killed by decapitation, and their livers were removed and weighed. Portions of the livers were homogenized in 9 or 18 volumes of 0·2 M-potassium phosphate buffer (pH 7·0) containing 1 mM-DTT. A teflon-glass homogenizer was used, and care was taken to homogenize the tissue without causing too much foaming. The tissue was disrupted with 10 'up and down' strokes of the rotating pestle. Homogenates were centrifuged at 100,000 g ($r_{av.}$ 6·4 cm) for 1 hour in a MSE Prepspin 50 centrifuge. The resulting supernatants were either used directly for enzyme assay, or were diluted with homogenization buffer prior to assay.

5. Assay of fatty acid synthetase activity

Fatty acid synthetase activity was determined by a spectrophotometric assay which measured malonyl-CoA-dependent NADPH oxidation. The rate of disappearance of NADPH, as measured by the decrease in absorbance at 340 nm, was followed by means of a Pye Unicam SP8-100 UV-vis spectrophotometer fitted with a chart recorder and a temperature probe. Enzyme assays were performed in semi-micro polystyrene cuvettes, with a path length of 1 cm, maintained at 30°C in the constant temperature cell housing of the spectrophotometer.

Unless stated otherwise, the assay system contained, in a final volume of 1 ml, 200 mM-potassium phosphate buffer (pH 7·0), 1 mM-DTT, 1 mM-EDTA, x µM-acetyl-CoA, y µM-malonyl-CoA, z µM-NADPH and enzyme (110 - 300 µg protein). The reaction was started by the addition of malonyl-CoA, and the rate of NADPH oxidation at 30°C was monitored at 340 nm for about 2 minutes. In each case, the initial slope of the recorder trace, which was linear with time, was used to calculate enzyme activity. Assays were performed in duplicate, using q and 2q volumes of enzyme preparation, to ensure linearity with respect to protein concentration. 'Blank' cuvettes contained 200 mM-potassium phosphate buffer (pH 7·0), 1 mM-DTT, 1 mM-EDTA, and varying aliquots of enzyme and distilled water to a final volume of 1 ml.

In order to maintain the reaction mixture at 30°C during the assay, it was necessary to pre-incubate a mixture (cocktail) of the potassium phosphate buffer, DTT and EDTA (in 0·8 ml) in a cuvette at 45°C for 5 minutes. After transfer to the spectrophotometer cell holder (set at 30°C), the other assay components (stored in ice) were added to give a total volume of 1 ml. Approximately 2 minutes after starting the reaction, the temperature in the cuvette, determined by the temperature probe, was found to be near 30°C. All reaction rates were corrected to

30°C using the following equation:-

$$\text{Log } Q_{10} = \frac{10 \ (\text{Log } K_1 - \text{Log } K_2)}{t_1 - t_2}$$

where K_1 and K_2 are the rates at the two temperatures t_1 and t_2, respectively.

One unit of fatty acid synthetase was defined in this study as the amount of enzyme required to catalyse the oxidation of 1 nmole NADPH per minute at 30°C.

6. Statistical analysis

Standard errors are provided to show the degree of variance in the data. Data were analyzed statistically by Student's 't' test, and levels of statistical significance are indicated where appropriate. Probability values (P) of 0·05 or less were considered to be significant.

RESULTS

1. Optimum conditions for the assay of fatty acid synthetase activity

Optimum conditions for the assay of fatty acid synthetase activity were determined for the enzyme extracted from the livers of untreated and oestrogenized chicks. Oestrogen-treated birds received an intramuscular injection of 1 mg 17β-oestradiol in propane-1,2-diol/100 g body weight 36 - 48 hours before death.

Initially, the conditions reported by Katiyar & Porter [494] for the assay of fatty acid synthetase from pigeon liver were adopted. The enzyme was assayed at pH 7·0, in the presence of 100 μM-NADPH, 15 μM-acetyl-CoA and 60 μM-malonyl-CoA. Each factor was then varied in turn,

keeping all other parameters constant, and the effect on enzyme activity was measured. As each factor was optimized, this value was inserted into the conditions used for subsequent determinations.

The optimum pH for the assay of fatty acid synthetase activity was found to be 7·0 (Fig. 25), in agreement with the results of Katiyar & Porter [494]. Maximum enzyme activity was obtained with NADPH concentrations of 80 - 200 µM (Fig. 26). Subsequent assays were conducted at pH 7·0 and contained 100 µM-NADPH. The concentrations of acetyl-CoA giving maximum enzyme activity were 15 - 30 (40) µM (Fig. 27). Subsequent assays contained 20 µM-acetyl-CoA. Malonyl-CoA concentrations of 30 - 40 µM produced optimum activity for the enzyme extracted from the livers of untreated chicks (Fig. 28(a)), and slight substrate inhibition was observed with higher concentrations of malonyl-CoA (up to 80 µM). This effect was not apparent for the enzyme extracted from the livers of oestrogen-treated birds (Fig. 28(b)), for which optimum activity was obtained with malonyl-CoA concentrations of 30 - 80 µM. Consequently, subsequent assays contained 30 µM-malonyl-CoA.

Oestrogen treatment did not significantly affect the optimum conditions obtained (Figs. 25 - 28). Therefore, all subsequent assays of the enzyme extracted from the livers of untreated, control and oestrogen-treated chicks were performed at pH 7·0, with final concentrations of 100 µM-NADPH, 20 µM-acetyl-CoA and 30 µM-malonyl-CoA.

2. The effect of a single intramuscular injection of 17β-oestradiol (1 mg/100 g body wt.) on the activity of hepatic fatty acid synthetase

In a preliminary experiment, the effect of a single intramuscular injection of 17β-oestradiol (1 mg/100 g body wt.) on the activity of hepatic fatty acid synthetase was examined at various times (6 - 96 h) after injection. In addition, one group of chicks received an injection

FIGURE 25

The effect of pH on the activity of fatty acid synthetase from livers of untreated and oestrogenized male chicks

Enzyme assays were performed at 30°C as described in the text. The enzyme was assayed at varying pH values, in the presence of 200 mM-potassium phosphate buffer (pH 6·2 - 8·0), 1 mM-DTT, 1 mM-EDTA, 100 µM-NADPH, 15 µM-acetyl-CoA and 60 µM-malonyl-CoA.

(a) Activity of fatty acid synthetase from untreated chick liver

(b) Activity of fatty acid synthetase from oestrogenized chick liver

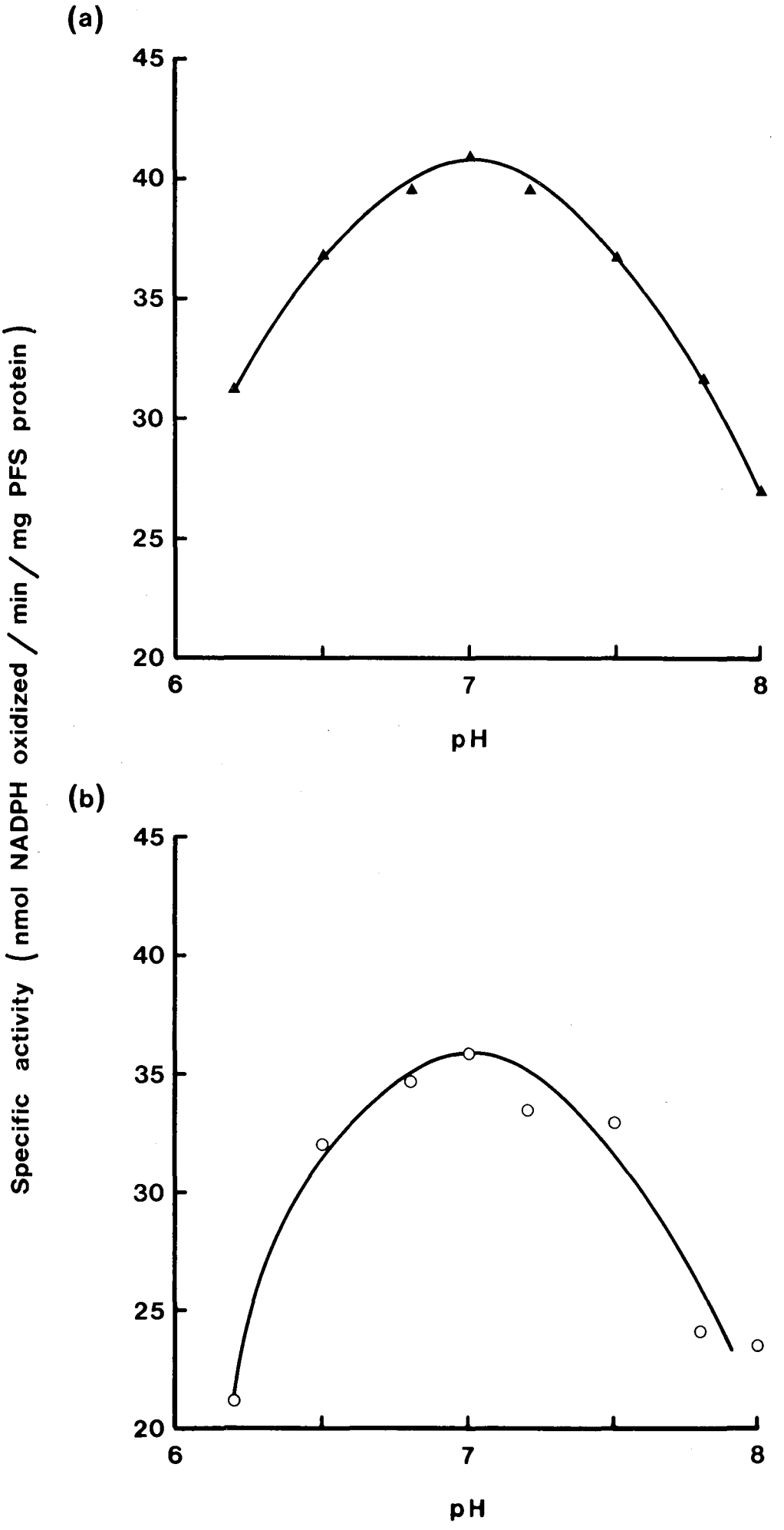

(a)
45
40
35
30
25
20
6
7
8
pH
(b)
45
40
35
30
25
20
6
7
8
pH
Specific activity (nmol NADPH oxidized / min / mg PFS protein)

FIGURE 26

The effect of NADPH concentration on the activity of fatty acid synthetase from livers of untreated and oestrogenized male chicks

Enzyme assays were performed at 30°C as described in the text. The enzyme was assayed in the presence of 200 mM-potassium phosphate buffer (pH 7·0), 1 mM-DTT, 1 mM-EDTA, 0 - 200 μM-NADPH, 15 μM-acetyl-CoA and 60 μM-malonyl-CoA.

(a) Activity of fatty acid synthetase from untreated chick liver

(b) Activity of fatty acid synthetase from oestrogenized chick liver

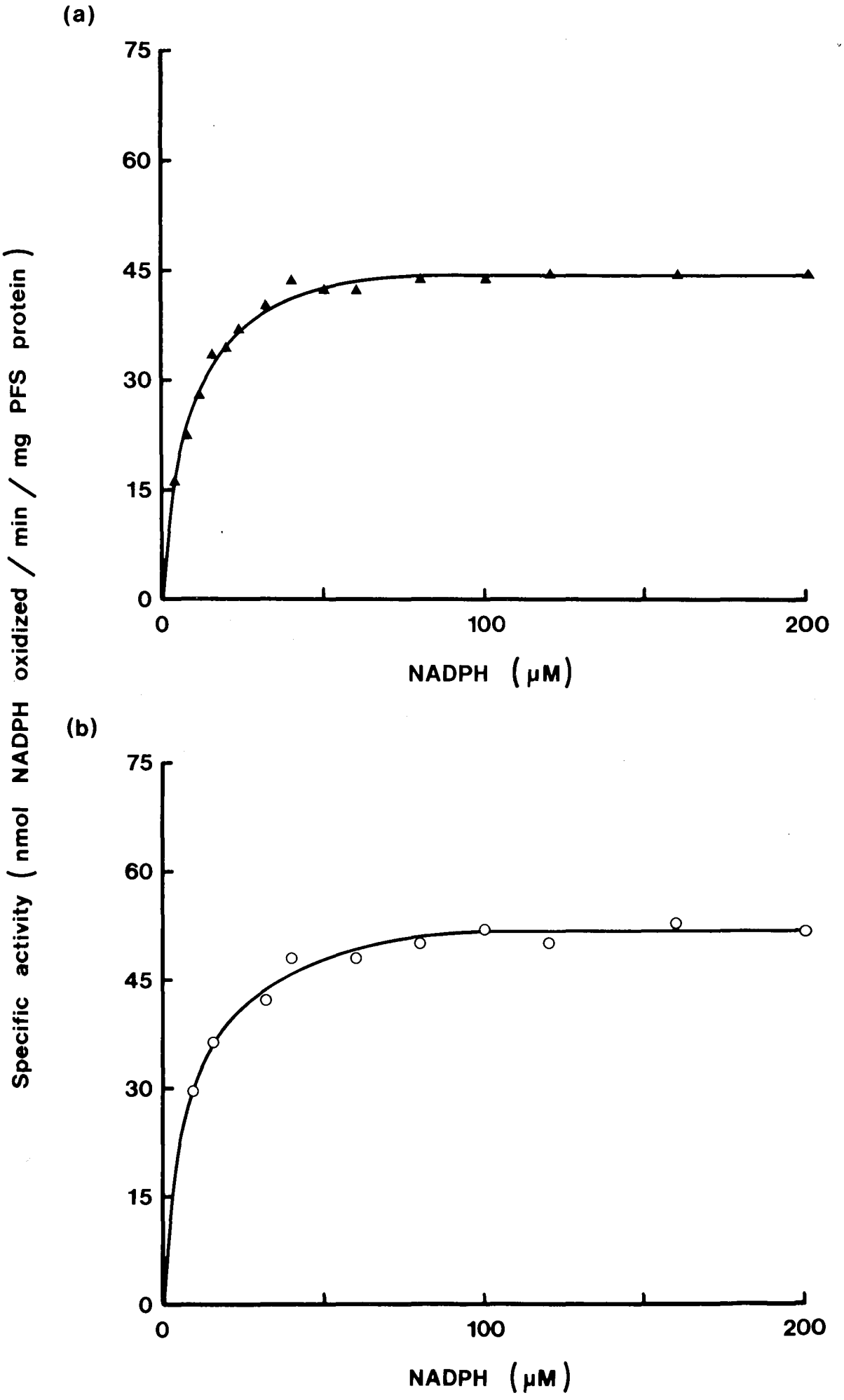

(a)
75
60
45
30
15
0
0
100
200
NADPH (μM)
Specific activity (nmol NADPH oxidized / min / mg PFS protein)
(b)
75
60
45
30
15
0
0
100
200
NADPH (μM)

FIGURE 27

The effect of acetyl-CoA concentration on the activity of fatty acid synthetase from livers of untreated and oestrogenized male chicks

Enzyme assays were performed at 30°C as described in the text. The enzyme was assayed in the presence of 200 mM-potassium phosphate buffer (pH 7·0), 1 mM-DTT, 1 mM-EDTA, 100 µM-NADPH, 0 - 40 µM-acetyl-CoA and 60 µM-malonyl-CoA.

(a) Activity of fatty acid synthetase from untreated chick liver

(b) Activity of fatty acid synthetase from oestrogenized chick liver

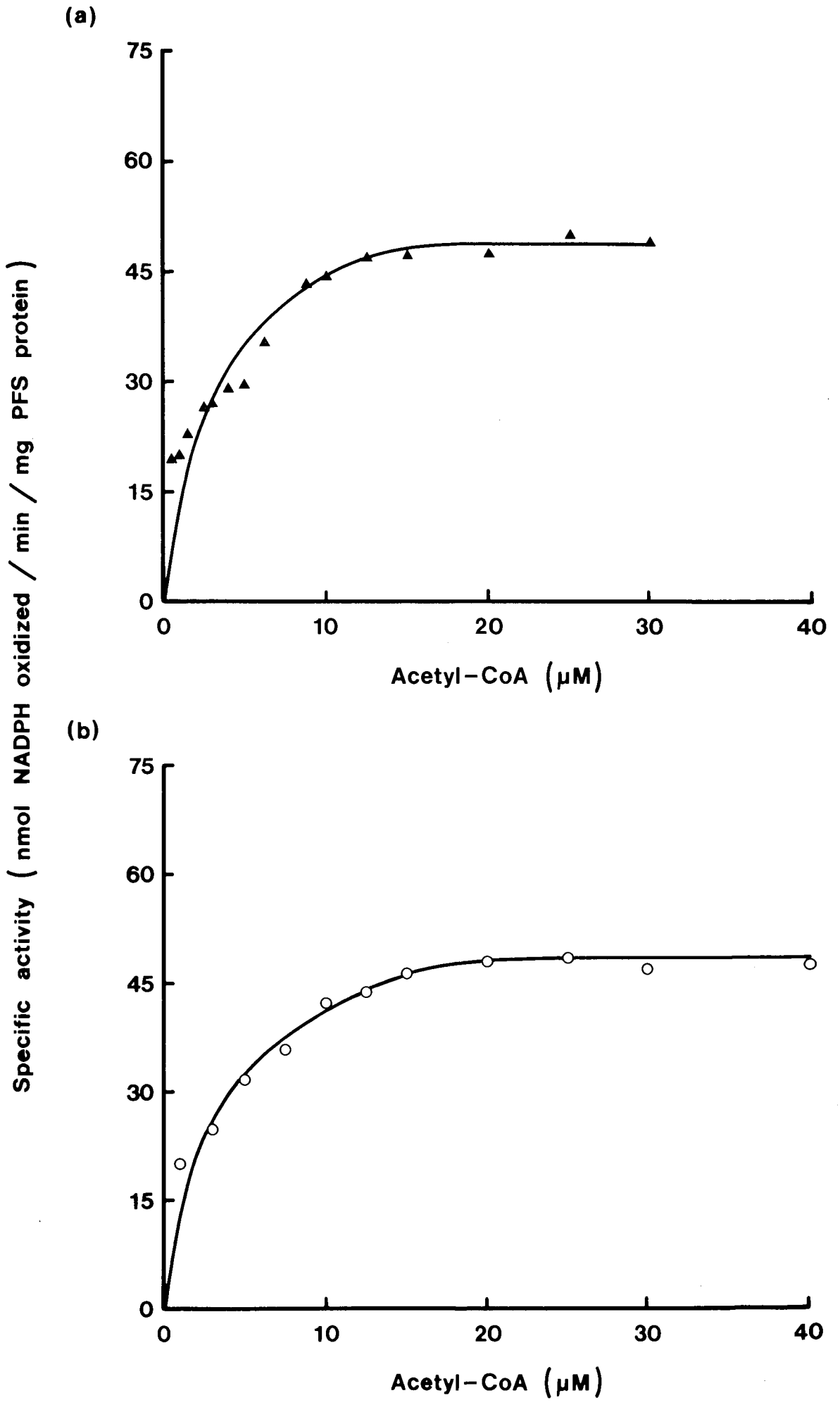

(a)
75
60
45
30
15
0
0
10
20
30
40
Acetyl-CoA (μM)
(b)
75
60
45
30
15
0
0
10
20
30
40
Acetyl-CoA (μM)
Specific activity (nmol NADPH oxidized / min / mg PFS protein)

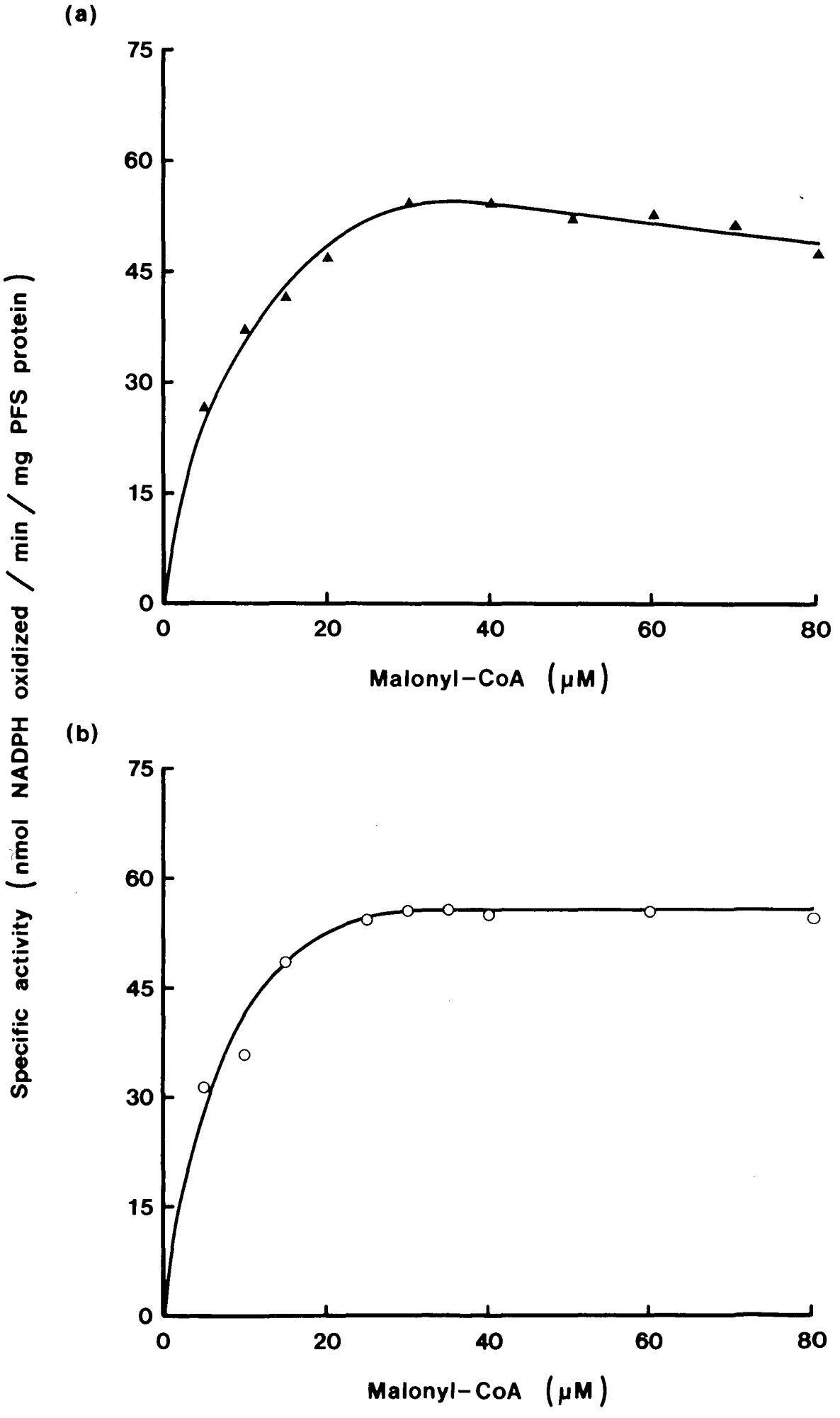
FIGURE 28

The effect of malonyl-CoA concentration on the activity of fatty acid synthetase from livers of untreated and oestrogenized male chicks

Enzyme assays were performed at 30°C as described in the text. The enzyme was assayed in the presence of 200 mM-potassium phosphate buffer (pH 7·0), 1 mM-DTT, 1 mM-EDTA, 100 μM-NADPH, 20 μM-acetyl-CoA and 0 - 80 μM-malonyl-CoA.

(a) Activity of fatty acid synthetase from untreated chick liver

(b) Activity of fatty acid synthetase from oestrogenized chick liver


(a)
75
60
45
30
15
0
0
20
40
60
80
Malonyl-CoA (μM)
(b)
75
60
45
30
15
0
0
20
40
60
80
Malonyl-CoA (μM)
Specific activity (nmol NADPH oxidized / min / mg PFS protein)

of 17β-oestradiol (1 mg/100 g body wt.) at 0, 24 and 48 hours, and these birds were sacrificed 24 hours after the last injection to determine the effect of long-term oestrogen administration. The results of this experiment are shown in Table 13 and Fig. 29. In this preliminary experiment, relatively few chicks were included in each treatment group, and large variations in enzyme activity were observed within each group, making it impossible to detect any significant changes in enzyme activity as a result of oestrogen treatment. The most notable observation resulting from this experiment, apart from the considerable variation in enzyme activity, was the apparent decrease in the soluble protein content of a unit weight of liver from oestrogenized chicks as compared with values for untreated and control chicks (Fig. 29(b)).

3. The effect of varying doses of 17β-oestradiol (0 - 1·25 mg/100 g body wt.) on the activity of hepatic fatty acid synthetase

It seemed pertinent to pursue this investigation further by varying the dose of 17β-oestradiol administered, in case the dose of 1 mg/100 g body weight used in the preliminary experiment was unsuitable to demonstrate effects on the activity of hepatic fatty acid synthetase. Each bird received a single intramuscular injection of 17β-oestradiol in propane-1,2-diol, equivalent to one of the following doses:-

0·25, 0·50, 0·75, 1·00, 1·25 mg 17β-oestradiol/100 g body weight

Control chicks, receiving no 17β-oestradiol, were injected with an equivalent volume of propane-1,2-diol only. Chicks were sacrificed 48 hours after injection, and the enzyme activities in the high-speed supernatants prepared from the livers were measured.

The results of this experiment are shown in Table 14 and Fig. 30. Again, there was considerable variation in enzyme activity between individual birds within each treatment group, and this is reflected in the

TABLE 13

The effect of 17β-oestradiol on the soluble protein content of chick liver and on the activity of hepatic fatty acid synthetase at various times after injection

The effect of a single intramuscular injection of 17β-oestradiol (1 mg dissolved in propane-1,2-diol/100 g body wt.) on the activity of hepatic fatty acid synthetase was examined at various times (6 - 96 h) after injection. To determine the effect of long-term oestrogen treatment, one group of chicks, (x 3), received an injection of 17β-oestradiol (1 mg/100 g body wt.) at 0, 24 and 48 hours, and these birds were sacrificed 24 hours after the last injection. The experiment included control chicks injected with propane-1,2-diol only, and untreated chicks.

Chicks were killed at the indicated times after injection, and enzyme preparations were obtained as described in the Methods section. Fatty acid synthetase activity in the high-speed supernatant was assayed at 30°C in the presence of final concentrations of 200 mM-potassium phosphate buffer (pH 7·0), 1 mM-DTT, 1 mM-EDTA, 100 μM-NADPH, 20 μM-acetyl-CoA and 30 μM-malonyl-CoA. The protein contents of the high-speed supernatants were assayed by a modified biuret method as described in the text.

The number of chicks in each group is shown in parentheses.

The results are expressed as means plus or minus half the range of equivalent determinations.

These results are illustrated graphically in Fig. 29(a - d).

Chicks were aged 3 – 5½ weeks.

Enzyme source	specific activity (nmol NADPH oxidized /min/mg PFS protein)	mg soluble protein /g liver	units of enzyme activity/g liver	units of enzyme activity/liver
Untreated chicks (4)	62·15 ± 14·58	110·2 ± 4·5	6836 ± 1534	101515 ± 26732
Control chicks				
6 h (2)	59·35 ± 6·39	105·5 ± 0·3	6260 ± 689	100902 ± 26190
12 h (2)	61·04 ± 0·04	106·4 ± 0·3	6492 ± 20	97921 ± 1296
18 h (2)	56·88 ± 4·76	107·0 ± 3·8	6104 ± 725	92271 ± 8792
24 h (5)	54·44 ± 14·76	103·3 ± 2·8	5633 ± 1670	65656 ± 50621
48 h (3)	61·04 ± 1·35	108·5 ± 4·4	6628 ± 413	48262 ± 3574
72 h (3)	73·35 ± 13·54	100·9 ± 5·1	7421 ± 1456	94146 ± 18615
96 h (3)	70·61 ± 1·60	100·7 ± 5·1	7114 ± 448	61087 ± 14939
x 3 (3)	49·09 ± 5·00	107·0 ± 2·4	5259 ± 645	60610 ± 5476
Oestrogenized chicks				
6 h (2)	72·41 ± 9·39	89·3 ± 8·8	6381 ± 205	106189 ± 4965
12 h (2)	51·43 ± 2·95	102·7 ± 4·3	5267 ± 84	77971 ± 7592
18 h (2)	48·01 ± 4·91	92·0 ± 4·8	4394 ± 222	78534 ± 11405
24 h (5)	41·72 ± 9·35	90·6 ± 12·1	3752 ± 776	53922 ± 28527
48 h (3)	61·67 ± 11·76	88·6 ± 12·1	5366 ± 410	76058 ± 24636
72 h (3)	45·34 ± 12·26	96·1 ± 2·4	4369 ± 1256	67070 ± 24967
96 h (3)	69·55 ± 14·53	92·4 ± 3·6	6390 ± 1095	83909 ± 19580
x 3 (3)	38·52 ± 10·53	91·2 ± 5·0	3479 ± 821	68348 ± 25987

FIGURE 29

The effect of 17β-oestradiol on the soluble protein content of chick liver and on the activity of hepatic fatty acid synthetase at various times after injection

Consult the legend of Table 13 for details of experimental procedure.

(a) specific activity (nmoles NADPH oxidized/min/mg PFS protein)

(b) mg soluble protein/g liver

▲ values for untreated chicks

● values for control chicks

○ values for oestrogen-treated chicks

The results are expressed as means plus or minus half the range of equivalent determinations.

FIGURE 29 (continued)

(c) units of enzyme activity/g liver

(d) units of enzyme activity/liver

▲ values for untreated chicks

● values for control chicks

○ values for oestrogen-treated chicks

The results are expressed as means plus or minus half the range of equivalent determinations.

TABLE 14

The effect of varying doses of 17β-oestradiol on liver weight, the soluble protein content of the liver and the activity of hepatic fatty acid synthetase 2 days after injection

Each oestrogen-treated chick received a single intramuscular injection of 17β-oestradiol in propane-1,2-diol, equivalent to one of the following doses:-

0·25, 0·5, 0·75, 1·0, 1·25 mg 17β-oestradiol/100 g body weight.

Control chicks received an equivalent volume of propane-1,2-diol only. Chicks were sacrificed 48 hours after injection, and liver enzyme preparations were obtained as described in the Methods section. Fatty acid synthetase activity in the high-speed supernatant was assayed at 30°C in the presence of final concentrations of 200 mM-potassium phosphate buffer (pH 7·0), 1 mM-DTT, 1 mM-EDTA, 100 μM-NADPH, 20 μM-acetyl-CoA and 30 μM-malonyl-CoA. The protein contents of the high-speed supernatants were assayed by a modified biuret method as described in the text.

Values are the means (± S.E.M.) for 6 chicks.

*These values are the means (± S.E.M.) for 5 chicks.

Data were analyzed statistically by Student's 't' test, and levels of statistical significance are presented with respect to values for both control and untreated chicks.

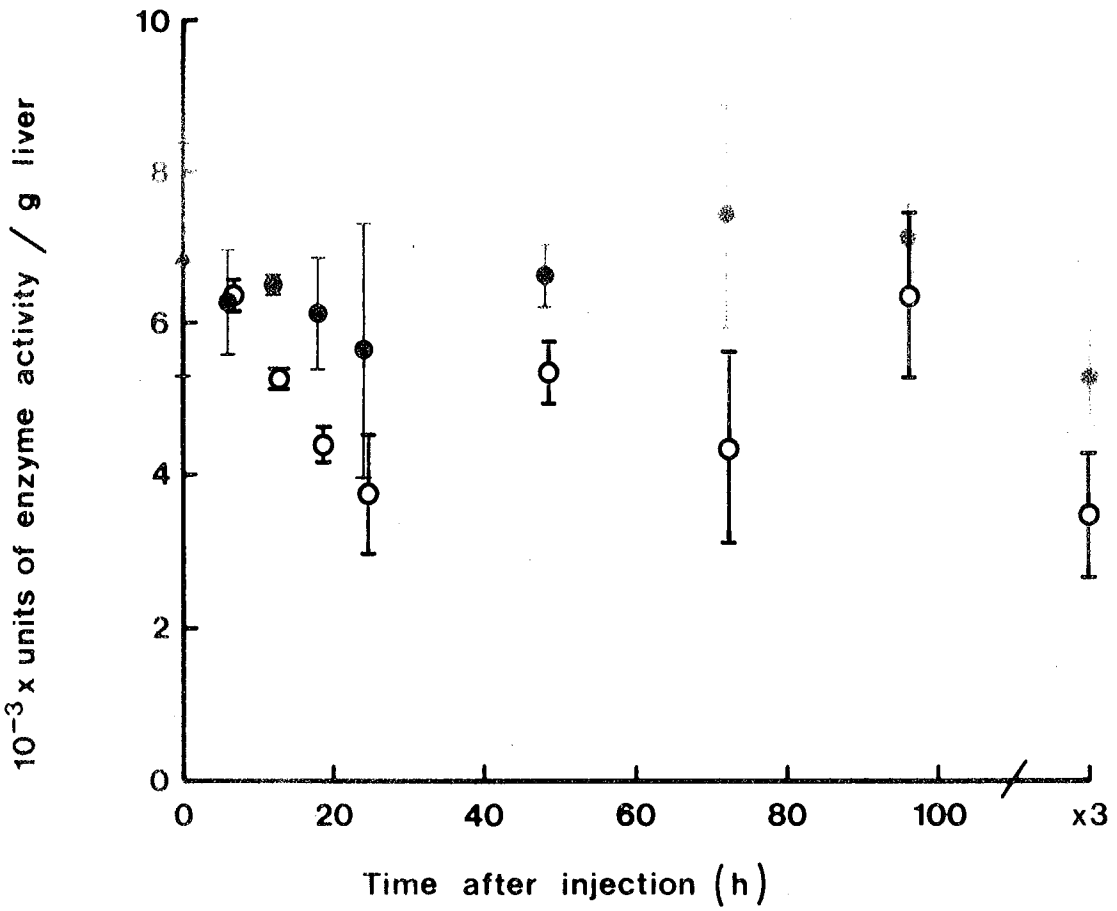
† significant at $P < 0·05$

‡ significant at $P < 0·02$

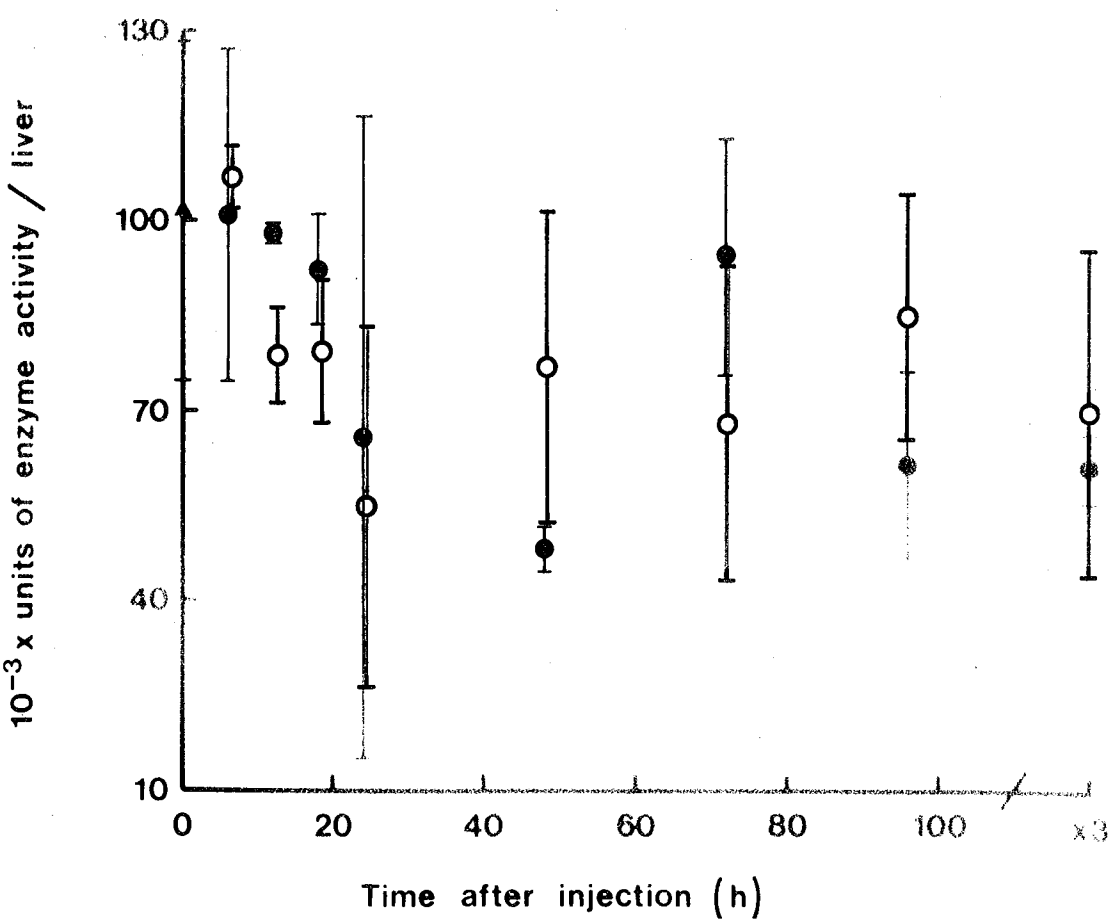
The enzyme activity results are illustrated graphically in Fig. 30(a - c).

Chicks were aged $4\frac{1}{2} - 5\frac{1}{2}$ weeks.

(c)


10
8
6
4
2
0
10^{-3} x units of enzyme activity / g liver
0
20
40
60
80
100
x3
Time after injection (h)

(d)


130
100
70
40
10
10^{-3} x units of enzyme activity / liver
0
20
40
60
80
100
x3
Time after injection (h)

	17β-oestradiol administered (mg/100 g body wt.)						
	0 Control	0 * Untreated	0·25	0·50	0·75	1·00	1·25
Body weight (g)	412 ± 15	395 ± 5	299 ± 7	348 ± 5	394 ± 17	396 ± 12	390 ± 22
Liver weight (as % of body wt.)	3·51 ± 0·22	3·46 ± 0·05	3·97 ± 0·07	4·42 ± 0·12	5·00 ± 0·14	5·43 ± 0·12	6·09 ± 0·21
Specific activity of fatty acid synthetase (units of activity/mg PFS protein)	38·80 ± 1·94	38·94 ± 2·96	42·90 ± 2·02	48·05 ± 4·48	50·01 ± 6·47	43·20 ± 3·63	40·81 ± 3·07
Amount of soluble protein/g liver (mg)	106·9 ± 1·8	109·9 ± 1·5	104·3 ± 0·9	112·1 ± 1·1	103·2 ± 2·4	101·2 ± 3·5	97·9 ± 2·9 †
Units of fatty acid synthetase activity/g liver	4156 ± 242	4287 ± 359	4467 ± 184	5396 ± 530	5147 ± 649	4333 ± 309	3962 ± 230
Units of fatty acid synthetase activity/liver	59087 ± 2307	58747 ± 5453	52987 ± 2510	83627 ± 9435	103873 ± 16171 †	94013 ± 9004 ‡	95850 ± 10986 ‡

The body weight and liver weight data have been presented in Table 2.

FIGURE 30

The effect of varying doses of 17β-oestradiol on the activity of hepatic fatty acid synthetase 2 days after injection

Consult the legend of Table 14 for details of experimental procedure.

▲ values for untreated chicks

● values for control chicks

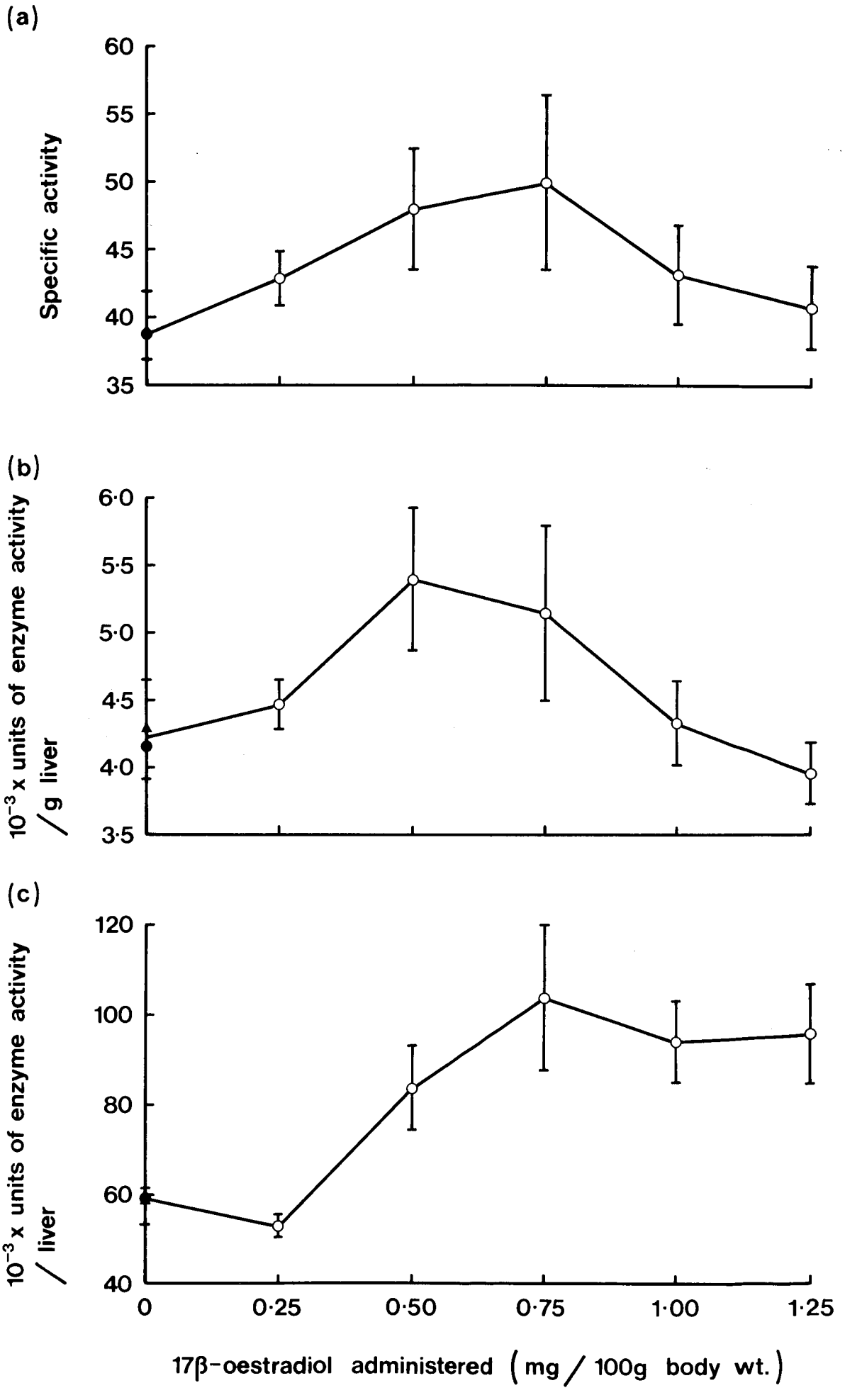
○ values for oestrogen-treated chicks

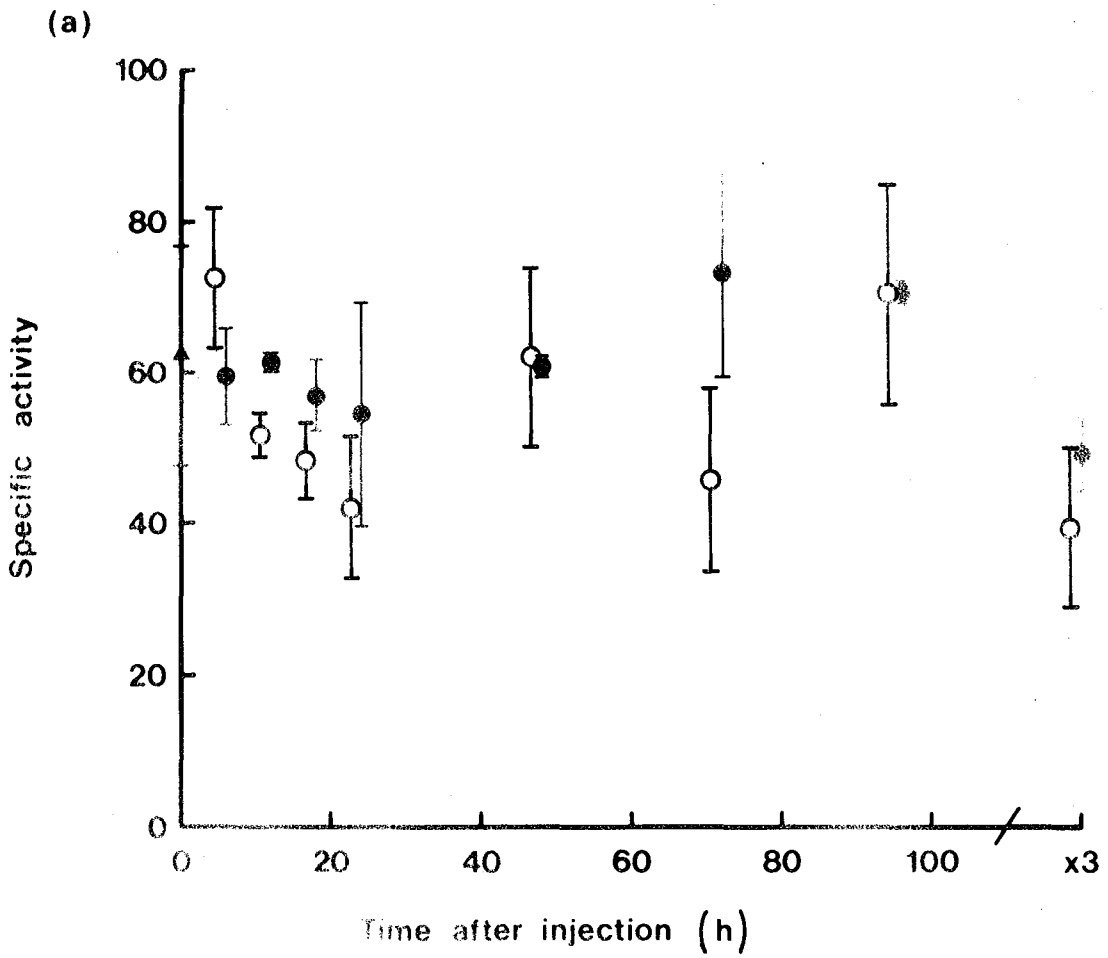
(a) specific activity (nmoles NADPH oxidized/min/mg PFS protein)

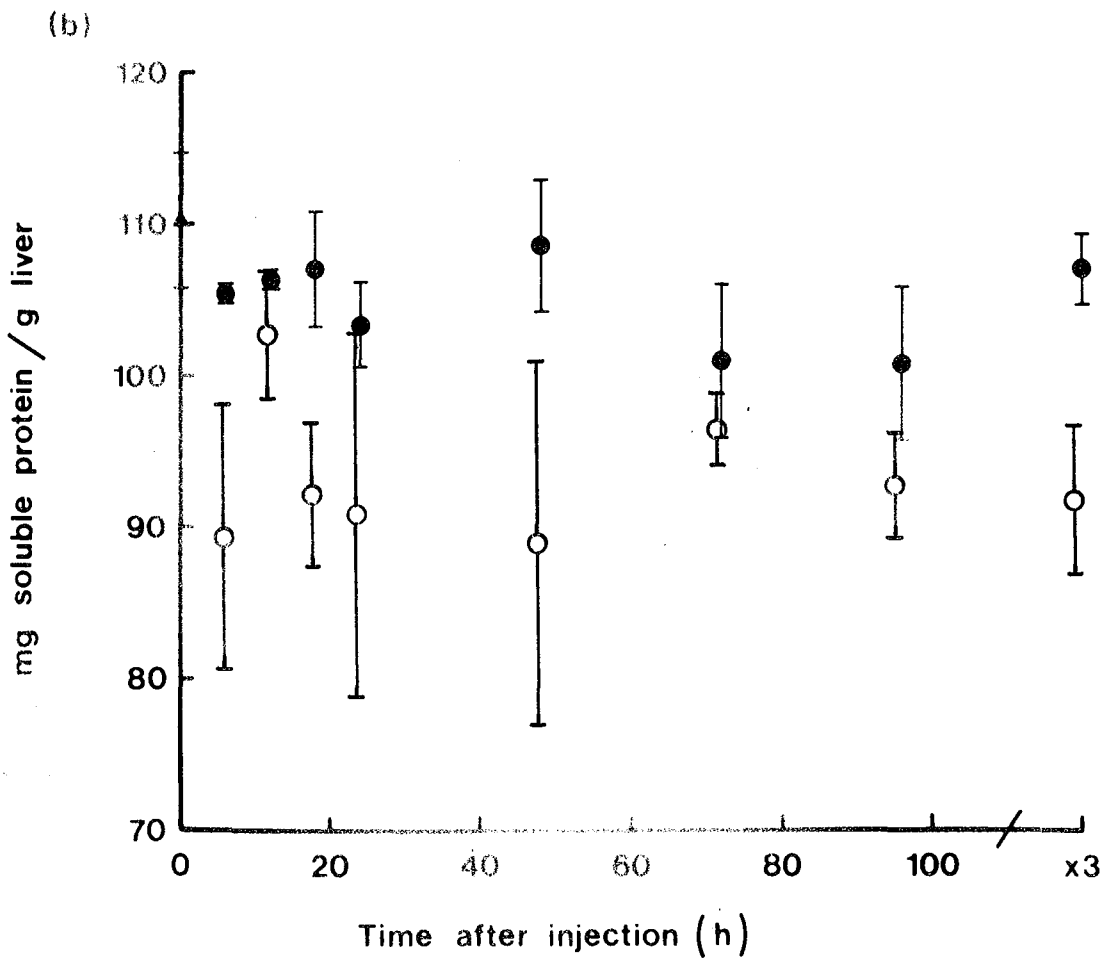
(b) units of enzyme activity/g liver

(c) units of enzyme activity/liver

The results are expressed as means (± S.E.M.) of 5 or 6 observations.


(a)
Specific activity
60
55
50
45
40
35
(b)
10^{-3} x units of enzyme activity / g liver
6·0
5·5
5·0
4·5
4·0
3·5
(c)
10^{-3} x units of enzyme activity / liver
120
100
80
60
40
0
0·25
0·50
0·75
1·00
1·25
17β-oestradiol administered (mg / 100g body wt.)


(a)
Specific activity
100
80
60
40
20
0
0
20
40
60
80
100
x3
Time after injection (h)


(b)
mg soluble protein / g liver
120
110
100
90
80
70
0
20
40
60
80
100
x3
Time after injection (h)

large S.E.M. values. The results indicate a slight increase in the mean specific activity of fatty acid synthetase as a result of oestrogen treatment, reaching a maximum with a dose of 0·75 mg hormone/100 g body weight, when the mean specific activity was approximately 29% higher than that of the control chicks (Fig. 30(a)). Higher doses of 17β-oestradiol (≧ 1 mg/100 g body wt.) caused a decrease in the mean specific activity of the enzyme, but at all hormone doses the mean specific activity was higher than the values for the control and untreated groups. Similar results were obtained when the data were expressed as enzyme activity/g liver, but in this case, the maximum mean activity occurred with a dose of 0·5 mg hormone/100 g body weight, and the mean activity with a dose of 1·25 mg hormone/100 g body weight was slightly lower than the mean values for the control and untreated birds (Fig. 30(b)). Statistical analysis of these results, using Student's 't' test, indicated no significant differences in specific activity (or activity/g liver) between control, untreated, and oestrogen-treated chicks at any dose of hormone administered, in this experiment.

Expression of enzyme activity as total organ activity revealed a slight decrease in mean total activity with the lowest dose of 17β-oestradiol (0·25 mg/100 g body wt.). This was followed by a substantial increase in mean total activity at higher doses. The mean total activity reached a maximum at 0·75 mg 17β-oestradiol/100 g body weight, when this value was approximately 76% higher than that of the control chicks (Fig. 30(c)). Further increases in 17β-oestradiol dose caused a slight decrease in mean total activity. Statistical analysis of these results indicated that oestrogen treatment caused a significant increase in total enzyme activity at all doses in the range 0·75 - 1·25 mg hormone/100 g body weight. The total organ enzyme activities after treatment with 17β-oestradiol, over the dose range 0·5 - 1·25 mg/100 g

body weight, did not differ significantly from each other. On the basis of these results, a dose of 0·75 mg 17β-oestradiol/100 g body weight was selected for future use, since this dose gave the maximum mean specific activity and maximum mean total activity in the experiment described above. It was noted that, with the exception of the result obtained with the 0·5 mg hormone dose, slight decreases in the mean soluble protein content of a unit weight of liver were observed for oestrogenized chicks (Table 14). The soluble protein content values for oestrogenized birds only attained significance with respect to the values for untreated and control birds with a dose of 1·25 mg hormone/100 g body weight. A dose-related increase in liver weight, as a percentage of body weight, was evident over the 17β-oestradiol dose range used in this experiment. This phenomenon has been discussed in Chapter 2.

4. The effect of a single intramuscular injection of 17β-oestradiol (0·75 mg/100 g body wt.) on the activity of hepatic fatty acid synthetase

The next experiment was conducted to investigate the effect of a single intramuscular injection of 17β-oestradiol (0·75 mg/100 g body wt.) on the activity of hepatic fatty acid synthetase at various times (3 - 40 h) after injection. The results are shown in Table 15 and Fig. 31. Hormone treatment caused a substantial increase in the mean specific activity of the enzyme at 3 and 6 hours after injection, compared with that of untreated chicks. Maximum mean specific activity was obtained 6 hours after hormone injection, when this value was more than double the value for untreated chicks (Fig. 31(a)). Following this, the mean specific activity declined sharply (at 14 hours post-injection), and then increased slowly over the next 26 hours. At all times after hormone injection up to 40 hours, the mean specific activity

TABLE 15

The effect of a single dose of 17β-oestradiol (0·75 mg/100 g body wt.) on liver weight, the soluble protein content of the liver and the activity of hepatic fatty acid synthetase at varying times after injection

Each oestrogen-treated chick received a single intramuscular injection of 0·75 mg 17β-oestradiol in propane-1,2-diol/100 g body weight. At the indicated times after injection (3 - 40 h) chicks were sacrificed, and liver enzyme preparations were obtained as described in the Methods section. Fatty acid synthetase activity in the high-speed supernatant was assayed at 30°C in the presence of final concentrations of 200 mM-potassium phosphate buffer (pH 7·0), 1 mM-DTT, 1 mM-EDTA, 100 μM-NADPH, 20 μM-acetyl-CoA and 30 μM-malonyl-CoA. The protein content of the high-speed supernatant was assayed by a modified biuret method as described in the text.

Values are the means (± S.E.M.) for 5 chicks.

Data were analyzed statistically by Student's 't' test, and levels of statistical significance are presented with respect to values for untreated chicks.

†	significant at $P < 0·05$
‡	significant at $P < 0·02$
††	significant at $P < 0·01$
Δ	significant at $P < 0·002$
▲	significant at $P < 0·001$

The enzyme activity results are illustrated graphically in Fig. 31(a - c).

Chicks were aged 4 – 5 weeks.

	Time after 17β-oestradiol injection (h)						
	0 Untreated	3	6	14	19	27	40
Body weight (g)	380 ± 36	290 ± 10	316 ± 14	405 ± 20	340 ± 14	385 ± 25	295 ± 10
Liver weight (as % of body wt.)	4·21 ± 0·47	4·23 ± 0·19	4·66 ± 0·17	4·43 ± 0·14	5·10 ± 0·27	5·23 ± 0·11	5·59 ± 0·30
Specific activity of fatty acid synthetase (units of activity/mg PFS protein)	33·79 ± 6·31	62·97 ± 4·13 ††	69·26 ± 3·09 ▲	41·85 ± 3·89	47·34 ± 2·46	46·96 ± 3·15	51·71 ± 1·87 †
Amount of soluble protein/g liver (mg)	104·0 ± 3·9	95·0 ± 2·2	88·7 ± 2·5 ‡	94·1 ± 3·1	83·5 ± 1·9 Δ	88·4 ± 2·1 ††	91·9 ± 1·1 ‡
Units of fatty acid synthetase activity/g liver	3584 ± 746	5971 ± 350 ‡	6131 ± 261 ‡	3917 ± 318	3948 ± 196	4162 ± 340	4758 ± 228
Units of fatty acid synthetase activity/liver	53309 ± 8768	73724 ± 7312	89624 ± 3909 ††	69859 ± 6455	67724 ± 2957	83015 ± 6436 †	77919 ± 4499 †

The body weight and liver weight data have been presented in Table 3.

FIGURE 31

Hepatic fatty acid synthetase activity at varying times after a single injection of 17β-oestradiol (0·75 mg/100 g body wt.)

Consult the legend of Table 15 for details of experimental procedure.

▲ values for untreated chicks

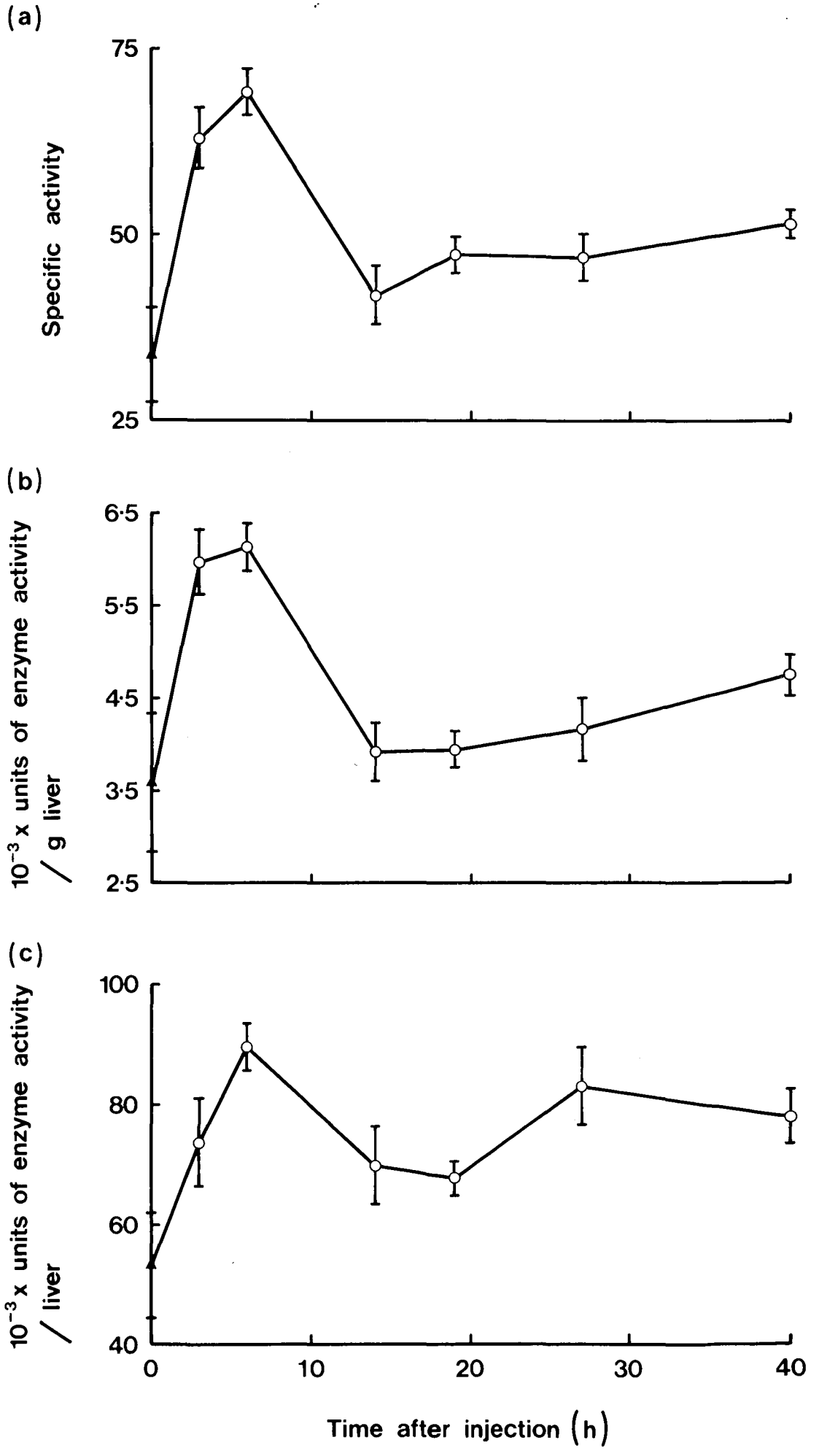
O values for oestrogen-treated chicks

(a) specific activity (nmoles NADPH oxidized/min/mg PFS protein)

(b) units of enzyme activity/g liver

(c) units of enzyme activity/liver

The results are expressed as means (± S.E.M.) of 5 observations.


(a)
75
50
25
Specific activity
(b)
6·5
5·5
4·5
3·5
2·5
10^{-3} x units of enzyme activity / g liver
(c)
100
80
60
40
10^{-3} x units of enzyme activity / liver
0
10
20
30
40
Time after injection (h)

was greater than that for untreated chicks. A similar response was observed when the enzyme activity was expressed as activity/g liver or as total organ activity (Fig. 31(b) & (c)). In both cases, maximum response was obtained 6 hours after hormone injection, when the mean values were approximately 70% higher than those of the untreated birds.

Statistical analysis of these results, using Student's 't' test, indicated significant differences in specific activity between untreated and oestrogenized chicks at 3, 6 and 40 hours after hormone injection (Table 15). When the enzyme activity was expressed as activity/g liver, significant increases were obtained at 3 and 6 hours. Total organ activities were significantly greater than those of untreated chicks at 6, 27 and 40 hours after oestrogen injection. The mean soluble protein content of a unit weight of liver for oestrogenized chicks, at all the time points studied, was noted to be lower than the value for untreated chicks. These hepatic soluble protein content values for oestrogenized chicks attained statistical significance with respect to the untreated chick values at 6, 19, 27 and 40 hours after hormone injection. With the exception of the value obtained at 14 hours, a time-related increase in liver weight, as a percentage of body weight, was observed up to 40 hours after 17β-oestradiol injection. This trend has been discussed fully in Chapter 2.

The lack of control chicks, injected with propane-1,2-diol only, in this latter experiment, raised the question that the enzyme activity differences observed may have been caused merely by handling and/or the injection of propane-1,2-diol, and not by the administered 17β-oestradiol. This possibility prompted the design of an experiment to include adequate controls. The effect of a single intramuscular injection of 17β-oestradiol (0·75 mg/100 g body wt.) on the activity of hepatic fatty acid synthetase was again investigated at various times

(3 - 26 h) after hormone injection. Control chicks, injected with an equivalent volume of propane-1,2-diol only, were included at each time point. The results of this experiment are shown in Table 16 and Fig. 32.

As in previous experiments, there was considerable variation in enzyme activity between individual birds within each treatment group. The results show an increase in the mean specific activity of hepatic fatty acid synthetase from oestrogenized and control chicks, above the value for untreated chicks, at all the time points studied. This indicates that handling and/or the injection of propane-1,2-diol was primarily responsible for the changes in specific activity observed. The mean specific activities of the enzyme from control and oestrogenized chicks were very similar up to 6 hours after injection, and maximum values were obtained at 14 hours. At this time, the mean values for control and oestrogenized chicks were approximately 50% and 40% higher, respectively, than that for the untreated chicks (Fig. 32(a)). A decrease in mean specific activity was observed at 26 hours, with the oestrogenized value declining to a greater extent than the control value. At 26 hours after injection, the mean specific activities of the enzyme from control and oestrogen-treated chicks were approximately 42% and 11% higher, respectively, than the mean value for untreated chicks. A similar pattern of response was observed when enzyme activity was expressed/g liver (Fig. 32(b)).

A slightly different response pattern was observed when the enzyme activity was expressed as total organ activity (Fig. 32(c)). In this case, the mean values for injected birds were once again greater than the mean value for the untreated birds. The mean total activity for oestrogen-treated chicks increased to a maximum at 6 hours after injection, when this value was approximately 37% higher than that of un-

TABLE 16

The effect of a single dose of 17β-oestradiol (0·75 mg in propane-1,2-diol/100 g body wt.), or of propane-1,2-diol only, on liver weight, the soluble protein content of the liver and the activity of hepatic fatty acid synthetase at varying times after injection

Each oestrogen-treated chick received a single intramuscular injection of 0·75 mg 17β-oestradiol in propane-1,2-diol/100 g body weight. Control chicks received an equivalent volume of propane-1,2-diol only. At the indicated times after injection (3 - 26 h) chicks were sacrificed, and liver enzyme preparations were obtained as described in the Methods section. Fatty acid synthetase activity in the high-speed supernatant was assayed at 30°C in the presence of final concentrations of 200 mM-potassium phosphate buffer (pH 7·0), 1 mM-DTT, 1 mM-EDTA, 100 μM-NADPH, 20 μM-acetyl-CoA and 30 μM-malonyl-CoA. The protein content of the high-speed supernatant was assayed by a modified biuret method as described in the text.

E = values for oestrogen-treated chicks

C = values for control chicks

Values are the means (± S.E.M.) for 5 chicks.

*These values are the means (± S.E.M.) for 4 chicks.

Data were analyzed statistically by Student's 't' test. Levels of statistical significance presented with values for oestrogenized chicks are with respect to corresponding control values. Levels of significance presented with values for control chicks are with respect to the values for untreated chicks.

† significant at $P < 0·05$

†† significant at $P < 0·01$

The enzyme activity results are illustrated graphically in Fig. 32(a - c).

Chicks were aged 4 – 5 weeks.

	* Untreated	Time after injection (h)							
		3		6		14		26	
		E	C	E	C	E	C	E	C
Body weight (g)	396 ± 9	368 ± 31	393 ± 16	405 ± 22	400 ± 22	320 ± 15	323 ± 20	344 ± 18	358 ± 7
Liver weight (as % of body wt.)	3·54 ± 0·06	4·00 ± 0·22	3·86 ± 0·13	4·01 ± 0·10	3·57 ± 0·11	4·54 ± 0·18	3·61 ± 0·06	5·03 ± 0·26	3·59 ± 0·17
Specific activity of fatty acid synthetase (units of activity/ mg PFS protein)	39·63 ± 1·16	43·84 ± 3·03	42·36 ± 2·12	47·80 ± 3·28	47·48 ± 3·63	55·33 ± 5·98	59·50 ± 4·47 ††	43·94 ± 4·17	56·24 ± 3·57 ††
Amount of soluble protein/g liver (mg)	107·0 ± 2·9	105·1 ± 2·7	105·7 ± 1·9	106·1 ± 3·1	106·6 ± 2·3	97·2 ± 3·5	105·0 ± 2·5	98·8 ± 2·7	108·8 ± 4·1
Units of fatty acid synthetase activity/g liver	4239 ± 153	4601 ± 304	4479 ± 245	5071 ± 373	5050 ± 370	5379 ± 612	6239 ± 465 ††	4323 ± 375 †	6159 ± 574 †
Units of fatty acid synthetase activity/liver	59709 ± 3987	66969 ± 7248	67769 ± 4320	81873 ± 6492	72689 ± 9174	76696 ± 6782	71917 ± 5065	76649 ± 11620	79618 ± 9766

The body weight and liver weight data have been presented in Table 4 .

FIGURE 32

Hepatic fatty acid synthetase activity at varying times after a single injection of 0·75 mg 17β-oestradiol in propane-1,2-diol/100 g body weight, or of propane-1,2-diol only

Consult the legend of Table 16 for details of experimental procedure.

▲ values for untreated chicks

● values for control chicks

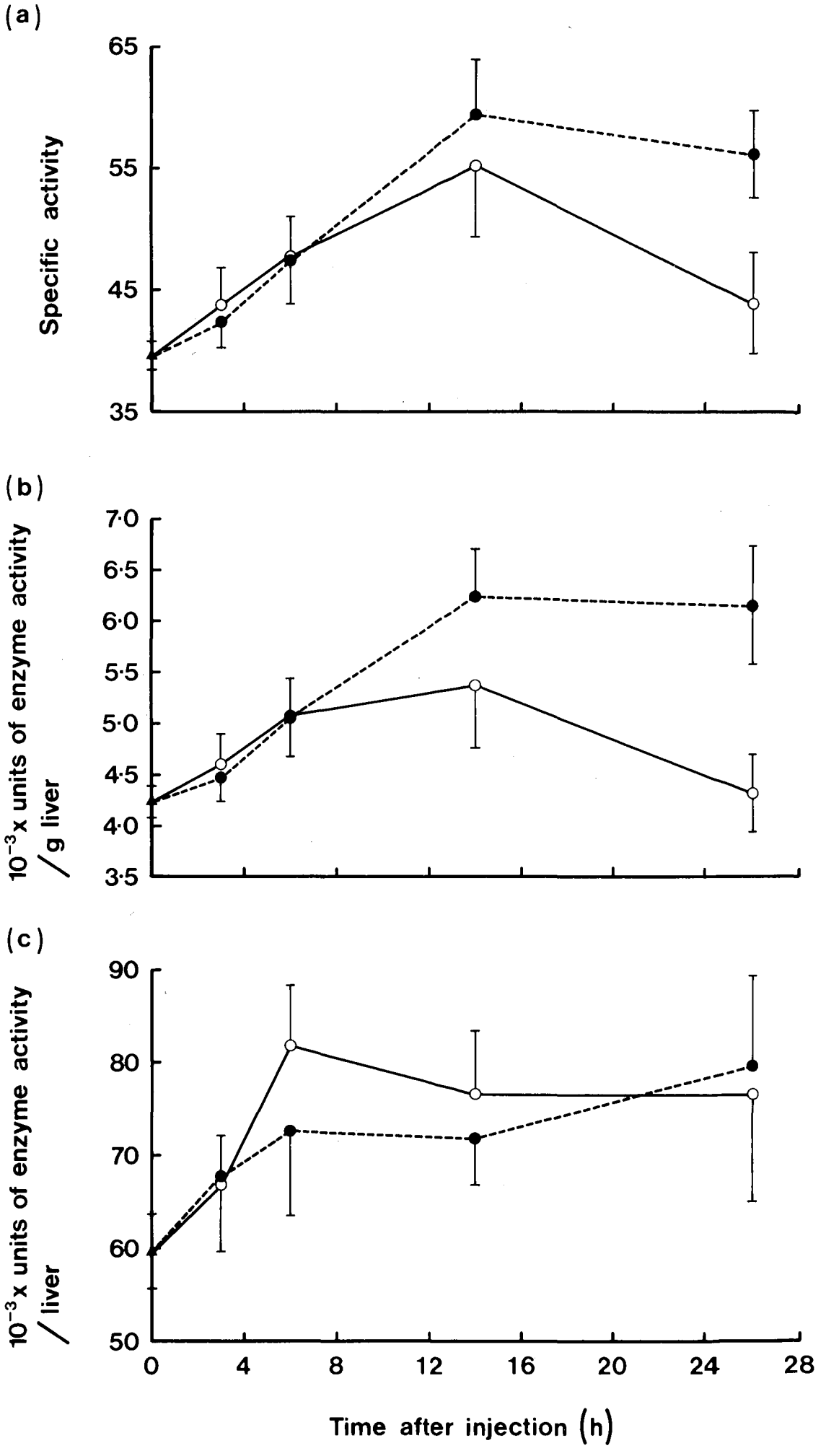
○ values for oestrogen-treated chicks

(a) specific activity (nmoles NADPH oxidized/min/mg PFS protein)

(b) units of enzyme activity/g liver

(c) units of enzyme activity/liver

The results are expressed as means (± S.E.M.) of 4 or 5 observations.


(a)
Specific activity
65
55
45
35
(b)
10^{-3}x units of enzyme activity / g liver
7·0
6·5
6·0
5·5
5·0
4·5
4·0
3·5
(c)
10^{-3}x units of enzyme activity / liver
90
80
70
60
50
0
4
8
12
16
20
24
28
Time after injection (h)

treated chicks, and then decreased slowly to a value at 26 hours which was approximately 28% higher than that of untreated chicks. On the other hand, the mean total activity for control chicks increased during the first 6 hours to a value which was approximately 22% higher than that of untreated chicks, and then increased to a maximum at 26 hours, when this value was 33% higher than that of untreated chicks.

Statistical analysis of these data showed that the specific activities of hepatic fatty acid synthetase from oestrogen-treated chicks did not differ significantly from the specific activities of the enzyme from their corresponding groups of control chicks, at any of the post-injection times studied. The specific activities for control chicks at 14 and 26 hours after injection were significantly greater than the specific activities for the untreated chicks (Table 16). When the enzyme activity was expressed/g liver, the values for oestrogenized chicks at 26 hours after injection were significantly lower than the values for the corresponding group of control chicks. As was the case with the specific activities, the activity/g liver values for control chicks at 14 and 26 hours after injection were significantly greater than the values for the untreated chicks. Total organ activities for the groups of oestrogen-treated chicks did not differ significantly from those of their corresponding control groups, and the values for the control groups did not differ significantly from those of the untreated chicks.

The mean soluble protein content/g liver was noticeably low for oestrogenized chicks at 14 and 26 hours after 17β-oestradiol injection, but these observations did not attain statistical significance. A similar decrease was not evident for the control birds. As noted previously, a time-related increase in liver weight, as a percentage of body weight, was observed after injection of 0·75 mg 17β-oestradiol/100 g

body weight (Table 16). This observation was evident as early as 3 hours after injection, although the mean values at 3 and 6 hours were virtually identical. The mean values at 14 and 26 hours were progressively larger. This response was not observed for the control chicks. This phenomenon has been discussed in Chapter 2.

DISCUSSION

1. Optimum conditions for assaying the activity of fatty acid synthetase from chick liver

Fatty acid synthetase from chick liver gave optimum activity when assayed at pH 7·0, with final concentrations of 100 µM-NADPH, 20 µM-acetyl-CoA and 30 µM-malonyl-CoA. These optimum conditions differ somewhat from the conditions employed by other workers to assay fatty acid synthetase from avian liver. Katiyar & Porter (494) demonstrated optimum activity of the enzyme from pigeon liver in the presence of 200 mM-potassium phosphate buffer at pH 7·0, with final concentrations of 100 µM-NADPH, 15 µM-acetyl-CoA and 60 µM-malonyl-CoA. Goodridge (468) performed assays of chicken liver fatty acid synthetase with 100 mM-potassium phosphate buffer (pH 7·0), and final concentrations of 180 µM-NADPH, 25 µM-acetyl-CoA and 100 µM-malonyl-CoA. Aprahamian *et al.* (31) employed 60 mM-potassium phosphate buffer at pH 6·5, and final concentrations of 112 µM-NADPH, 20 µM-acetyl-CoA and 60 µM-malonyl-CoA, to assay the enzyme from chicken liver and oviduct. Fatty acid synthetase from pigeon liver has been shown to be very susceptible to loss of activity in low ionic strength buffer (501), and therefore, 200 mM-potassium phosphate buffer was employed in the present study. Clearly, the principal difference between the conditions used in the present study and those referred to above lies in the concentration of malonyl-CoA

employed in the assay. In the present investigation, high concentrations of malonyl-CoA (> 40 μM) were found to give a small but progressive inhibition of the enzyme extracted from the livers of untreated chicks. A similar effect, and also inhibition by acetyl-CoA, have been reported by other workers, and it has been shown that acetyl-CoA and malonyl-CoA exhibit a competitive inhibition with respect to each other [488, 492, 494, 495, 502]. Consequently, a concentration of 30 μM-malonyl-CoA was selected for subsequent assays. In addition, the finding that oestrogen treatment of chicks *in vivo* did not affect the optimum assay conditions of the enzyme *in vitro*, meant that all subsequent assays of the enzyme could be performed under the same optimum conditions.

2. The effect of varying doses of 17β-oestradiol (0 - 1·25 mg/100 g body wt.) on the activity of hepatic fatty acid synthetase

Variation in the dose of 17β-oestradiol administered to chicks *in vivo*, within the range 0·25 - 1·25 mg hormone/100 g body weight, caused moderate increases in the mean specific activity of fatty acid synthetase in liver cell extracts prepared 48 hours after hormone administration. A similar response pattern was observed when enzyme activity was expressed as total organ activity, although a slight decrease in the mean value was observed with a dose of 0·25 mg when compared with the mean values for control and untreated chicks.

Mean specific activity and mean total activity reached maximum values with a dose of 0·75 mg hormone/100 g body weight. These values corresponded to a 29% increase in mean specific activity and a 76% increase in mean total activity compared with the control values. Only the total activity values were found to differ significantly from those of the control and untreated chicks. With a dose of 0·75 mg hormone/100 g body weight, the mean liver weight (as % of body wt.) increased

by 42 - 45%. Under the same conditions, the soluble protein content/g liver did not alter significantly. These results suggest that the significant increase in total fatty acid synthetase activity may be due largely to new enzyme activity appearing concurrently with organ 'growth'.

Higher doses of 17β-oestradiol (1 & 1·25 mg hormone/100 g body wt.) caused a slight decrease (approx. 8 - 9%) in mean total activity compared with that observed with the 0·75 mg dose. Increases in mean liver weight (as % of body wt.) were obtained with doses of 1 and 1·25 mg hormone, for which values were 9% and 22% higher, respectively, than that obtained with the 0·75 mg dose. A significant decrease in soluble protein content/g liver was obtained with a dose of 1·25 mg hormone. With doses of 1 mg and 1·25 mg hormone, there were decreases in mean specific activity and mean activity/g liver. These observations suggest that, at higher oestrogen levels, organ 'growth' is accompanied by accumulation of proteins other than fatty acid synthetase, and also by decreased synthesis and/or increased degradation of selected proteins including fatty acid synthetase.

Pearce & Balnave [256] reported similar effects of variations in oestrogen dose on the activities of ATP citrate lyase and 'malic' enzyme in liver extracts from immature pullets 2 days after oestrogen treatment. Using oestradiol dipropionate, these workers reported maximum specific activities of these enzymes with 2 mg hormone/bird and 1 mg hormone/bird, respectively. Thus, after correction for body weight and quantity of oestradiol, the maximum specific activities were obtained with approximate doses of 0·57 mg oestradiol/100 g body weight for ATP citrate lyase, and 0·28 mg oestradiol/100 g body weight for 'malic' enzyme. In the present investigation, therefore, maximum specific activity of fatty acid synthetase was observed at a higher dose of oestradiol (0·75 mg/100 g body wt.) than reported for ATP citrate lyase

and 'malic' enzyme. This difference in dose levels may be the result of sex differences, since male chicks were used throughout the present study, whilst Pearce & Balnave [256] used immature females. It is possible that immature pullets at 4 weeks of age possess endogenous oestrogen levels capable of exerting effects on enzyme activity, and/or higher levels of oestrogen receptor proteins in the liver cells than do male chicks. In addition, different enzymes may have different susceptibilities to oestradiol treatment.

3. The effect of a single intramuscular injection of 17β-oestradiol (0·75 mg/100 g body wt.) on the activity of hepatic fatty acid synthetase

Pearce & Balnave [256] observed reduced specific activities of ATP citrate lyase and 'malic' enzyme after administering oestrogen to immature pullets over a period of 8 days, and consequently recommended that short-term investigations, within 2 days of hormone administration, would be more indicative of induced physiological effects, since pharmacological effects might predominate after longer times. As the initial enzyme changes after oestrogenization were of major interest in the present study, investigations were performed within 2 days of hormone treatment. The activity of hepatic fatty acid synthetase, determined at various times (3 - 40 h) after a single intramuscular injection of 17β-oestradiol (0·75 mg/100 g body wt.), was considerably increased (Fig. 31). The maximum mean specific activity and mean total organ activity were observed at 6 hours after 17β-oestradiol injection. At this time, the mean specific activity was more than double that of the untreated chicks, whilst the mean total activity was approximately 68% higher than that of the untreated chicks.

At 6 hours after hormone injection, values for the soluble protein content/g liver were significantly lower than values from untreated

chicks, and the mean liver weight (as % of body wt.) was approximately 11% greater than the value for untreated chicks. These results suggest that the increased liver weight at this early time point may be the result of cell expansion caused by water uptake, rather than, or in addition to, increased cell growth with concomitant protein synthesis. The significant increases in specific activity at 3 and 6 hours post-injection indicate that a greater proportion of soluble cell protein exhibits fatty acid synthetase activity at these time points. The significant increase in total fatty acid synthetase activity at 6 hours after injection implies new enzyme activity appearing simultaneously with the increase in organ size. The decrease in mean specific activity observed at 14 hours is accompanied by a decrease in mean total activity, but both of these values increase slowly again over the following 26 hours.

The values for soluble protein content/g liver at 19, 27 and 40 hours after 17β-oestradiol injection were significantly lower than untreated chick values, and the mean specific activities at these time points were considerably lower than the mean value at 6 hours. These results indicate that, at these later time points after oestrogenization, a lesser proportion of soluble cell protein exhibits fatty acid synthetase activity than at the 6-hour time point. The data illustrated in Fig. 31(c) show that the total enzyme activity does not continue to increase as liver size increases.

Since control chicks, receiving the injection vehicle only, were not included in this experiment, it was not possible to distinguish the cause of the changes in enzyme activity. The fact that substantial activity changes were obtained at such short time intervals after oestrogen injection (3 & 6 h), posed the suggestion that stress might have contributed to the changes. The experiment illustrated in Fig. 32

incorporated control chicks, injected with propane-1,2-diol only, at each of the post-injection time points studied. Again, oestrogen-treated chicks exhibited increased mean specific activities at all time points up to 26 hours after injection, but a similar response was also observed with control chicks. Maximum mean specific activities for both control and oestrogenized chicks were obtained at 14 hours after injection, which is rather later than the peak in mean specific activity at 6 hours observed in the previous experiment. The specific activities for control chicks at 14 and 26 hours after injection were the only specific activity values to attain significance with respect to the values for untreated chicks. The mean specific activities for control and oestrogenized chicks were very similar up to 6 hours after injection, but at 14 hours the value for control chicks was approximately 7·5% higher than that for the corresponding group of oestrogenized chicks. At 26 hours, the mean specific activity for control chicks was approximately 28% higher than that for oestrogenized chicks at this time.

The sharper decline in the mean specific activity of hepatic fatty acid synthetase for oestrogen-treated chicks than for control chicks, from 14 to 26 hours, may be the result of end product inhibition of the enzyme from oestrogen-treated chicks by accumulating lipid. It is well-known that after oestrogen treatment *in vivo*, the chicken liver becomes fatty and lipaemia develops, and this has been shown to be evident at 14 hours after hormone administration (see Chapter 2). This increase in lipid production is undoubtedly the result of increased hepatic lipogenic enzyme activities, brought about either by increases in the concentrations of enzymes or by the activation of constant amounts of enzymes. Fatty acid synthetase may, or may not, be involved in these enzyme changes after oestrogen treatment, but the resulting accumulating lipid may have an inhibitory effect on fatty acid synthetase and other lipo-

genic enzyme activities. Such inhibition may be the cause of the sharp decline in mean specific activity (and mean activity/g liver) observed in Fig. 32. Since control chicks do not develop fatty liver and lipaemia after propane-1,2-diol injection, no such inhibitory effect would be expected for control chicks, and this is substantiated by only a small decline in mean specific activity (and mean activity/g liver) from 14 to 26 hours.

The maximum mean total activity for the enzyme of oestrogen-treated chicks was observed at 6 hours, as was the case in the previous experiment (Fig. 31(c)). Inhibitory effects may be operative, as described above, to cause the decrease in mean total activity after this time. This 6-hour value for oestrogenized chicks was only slightly higher than the maximum mean total activity attained at 26 hours for control chicks. These data imply that handling and/or the injection of propane-1,2-diol may be the major cause of the increases in enzyme activity observed up to 26 hours after injection. This is of particular interest when compared with the results presented in Table 10 in Chapter 3, in which the incorporation of tritium from ^3H_2O into total lipid was measured in chick liver slices. In those experiments, the incorporation values for control chicks, that had received an injection of propane-1,2-diol $3\frac{1}{2}$ - $7\frac{1}{4}$ or 14 - 17 hours before death, were significantly greater than the values for untreated chicks. This suggests that fatty acid synthesis was stimulated at these early times by handling and/or the injection of propane-1,2-diol. This may, however, be a short-term phenomenon, since it has been shown that 48 hours after injection greater hepatic fatty acid synthetase activities are observed for oestrogen-treated chicks than for control chicks (Fig. 30), and the ^3H_2O incorporation values for control chicks at times later than 17 hours after injection were not significantly different from untreated values (Table 10). These data, there-

fore, suggest that an oestrogen-dependent increase in fatty acid synthetase activity occurs at some point between 26 and 48 hours after injection of 17β-oestradiol (0·75 mg/100 g body wt.).

In this experiment (Table 16), no significant differences in soluble protein content/g liver were obtained, although noticeably lower mean values were observed at 14 and 26 hours after oestrogenization. As in the previous experiment, a time-related increase in liver weight (as % of body wt.) was evident as early as 3 hours after 17β-oestradiol injection. No such response was observed for control chicks at any time point, confirming that this phenomenon is the result of oestrogen treatment and not of handling and/or the injection of propane-1,2-diol.

The results of this latter experiment throw some doubt upon the conclusions drawn from the data of Pageaux et al. [235]. These workers reported a rapid increase in hepatic acetyl-CoA carboxylase activity after treating 16-day old female quail with a single injection of oestradiol benzoate (0·2 mg/kg body wt.). A peak in specific activity was reached at 3 hours after injection, and the values at 3 and 6 hours were significantly higher than 'control' values. The hormone dose used by these workers was extremely low compared with the dose levels administered to birds by other workers [31, 256] and those used in the present study. At 3 hours after oestrogen injection, Pageaux et al. [235] observed a 37% increase in mean acetyl-CoA carboxylase specific activity compared with that of 'control' birds. However, the description of the experimental procedure suggests that the 'control' birds were injected with the olive oil vehicle immediately before death, and were not sacrificed at the same post-injection times as the oestrogenized birds. It appears, therefore, that adequate controls were not performed, so that effects due to stress or administration of the injection vehicle could not be excluded. As a result of their experimental design, data ob-

tained from the 'control' birds used by Pageaux et al. [235] may be comparable with those obtained from the untreated chicks in the present study. The values obtained by Pageaux et al. [235] at 1 hour and 24 hours after hormone injection were very similar. The description of the experimental procedure implies that these 2 groups of birds were sacrificed at approximately the same time on consecutive days. The enzyme activity variations obtained at the other time points may, therefore, have been influenced by the time of day. In the present study, chicks were killed at or near midday whenever possible, so that any diurnal effects did not obscure the investigation.

Philipp & Shapiro [140] have measured acetyl-CoA carboxylase and fatty acid synthetase activities in liver extracts from oestrogenized male Xenopus laevis. These animals received a 17β-oestradiol injection (0·4 mg/100 g body wt.) at 0 and 24 hours, and were sacrificed for enzyme assay over an extended period (1 - 14 days). Oestrogen treatment resulted in increased hepatic fatty acid synthesis and a concurrent increase in acetyl-CoA carboxylase activity, with peaks in both of these parameters on the 6th day. No change in fatty acid synthetase activity was observed during their study. However, another substantial increase in fatty acid synthesis was observed from the 8th to the 13th day of the experiment, without a concomitant increase in acetyl-CoA carboxylase activity. This observation, together with the small number of animals examined (2 at each time point) and the lack of control values for comparison, casts some doubt on the conclusions drawn from the data by these workers about the action of the oestrogen.

Aprahamian et al. [31] examined hepatic fatty acid synthetase activity after treating 1-month old pullets with oestrogen. After prolonged oestrogenization (4 mg β-oestradiol-3-benzoate/bird/day for 6 days), a 3-fold increase in total organ enzyme activity was observed.

This increase was accompanied by a 2-fold increase in specific activity and an approximately 66% increase in liver weight. These workers also assayed the fatty acid synthetase from chicken oviduct, and found an enormous increase in total and specific activity of the enzyme in this organ after oestrogenization. The total enzyme activity of the oviduct increased 180-fold, whilst that of the liver only tripled after oestrogen treatment. However, it should be noted that the total activity of fatty acid synthetase in the liver was approximately 150-fold higher than that in the oviduct. The lipid produced by the oviduct undoubtedly passes into the egg, but it is well-documented that the liver makes a considerable contribution to the lipids of the developing ovum. Therefore, as regards the lipid requirements of the egg, the increase in the fatty acid synthetase activity of the liver would appear to be quantitatively more significant than the increase in enzyme activity of the oviduct.

A decrease in soluble protein content/g liver is evident from the work of Aprahamian et al. [31], and a similar decrease has been observed during the present study. The specific activities obtained in the current work are greater than those reported by Aprahamian et al. [31] for chicken liver fatty acid synthetase. These workers reported a specific activity of about 5 nmoles NADPH oxidized/min/mg protein for control hen liver enzyme, which was doubled after oestrogen treatment. Several factors may be responsible for the discrepancies between the values from this work and those obtained in the present investigations. Notable differences are evident in the preparation of the enzyme, in substrate concentrations, and in the concentration of the potassium phosphate buffer and pH of the assay. Aprahamian et al. [31] used a lower temperature (23 - 26°C) for assaying the enzyme, and a different method of protein estimation based on absorbance at 280 nm. In the

present study, the tissue was homogenized in large volumes of buffer, and it may be that an inhibiting factor was diluted out in this procedure, contributing to the high specific activities observed. Goodridge (424) recorded a mean specific activity of about 52 nmoles NADPH oxidized/min/mg protein for fatty acid synthetase from 16-day old male and female chick livers. However, the temperature of assay (40°C) was very much higher than that used in the present study. Similarly, Muiruri et al. (46) assayed fatty acid synthetase at 37°C, and reported a mean specific activity of 43 nmoles NADPH oxidized/min/mg protein for the hepatic enzyme from ad libitum-fed male and female chickens. In addition to the temperature differences, the studies of Goodridge (424) and Muiruri et al. (46) differ from each other and from the present study in the enzyme preparation and assay conditions used, and therefore the results are not directly comparable.

Measurements of enzyme activities in subcellular preparations under optimum conditions give an estimate of the total activity potential in the tissue. These conditions may not exist in vivo, and the enzyme may be affected in the cell by substrate deprivation or by regulatory metabolites. Changes in the activity of fatty acid synthetase could be the result of alterations in the catalytic efficiency of existing enzyme protein, or of variations in the amount of enzyme protein caused by changes in the rate of enzyme synthesis and/or degradation.

In general, steroid hormones act by stimulating the transcription of selected genes, causing increased synthesis of selected enzymes and other proteins (130 - 132). This mechanism may account for changes in enzyme activity observed after oestrogen treatment of birds and Xenopus laevis (31, 140, 235, 256). Decreases in enzyme activity at longer times after oestrogenization (Fig. 32) (166, 256) could be the result of a decrease in the amount of enzyme protein or of end product

inhibition of enzyme activity by accumulating lipid. The present experiments, however, do not allow us to distinguish between these alternative mechanisms.

CHAPTER 5

GENERAL DISCUSSION AND SUGGESTIONS FOR FURTHER WORK

GENERAL DISCUSSION

1. The use of model systems for studying vitellogenesis in the domestic fowl

Increases in the levels of vitellogenin and VLDL in blood, which are observed as the female domestic fowl approaches the point of lay, are thought to be due primarily to the oestrogen-dependent stimulation of lipid and protein biosynthesis in the female liver. This situation can be simulated in male or sexually-immature female birds by administration of exogenous oestrogen *in vivo*, providing a convenient model system for the investigation of the molecular events involved in these changes. In particular, male birds, with no endogenous oestrogen, have been used extensively to study the oestrogen-dependent stimulation of the hepatic biosyntheses of VLDL [113 - 119, 206] and vitellogenin [81, 141, 162, 163].

The physiological state of the mature laying hen is, however, undoubtedly under the influence of a number of internal secretions in addition to oestrogen. The administration of oestrogen to male and immature female birds, therefore, cannot be expected to completely reproduce the natural state of fatty acid metabolism existing in the laying hen. Several notable differences in lipid metabolism between oestrogen-treated birds and laying hens have been reported in the literature, although these may simply be the result of the high oestrogen doses administered compared with the endogenous oestrogen levels that exist in the hen. For example, Balnave [164] observed heavier livers and higher liver and blood levels of fatty acids for immature pullets treated with oestrogen than for mature laying hens. In addition, the retention of dietary linoleic acid is increased at the onset of sexual maturity in the female bird [164], whilst the administration of oestrogen to immature pullets has no effect on dietary linoleic acid retention [503]. Significant increases in the percentage of palmitic acid in blood and

liver fatty acids have been observed for oestrogen-treated pullets but not for the mature hen, suggesting that palmitic acid might be metabolized abnormally in the oestrogen-treated pullet [164, 464]. It is interesting to note that palmitic acid is the principal fatty acid synthesized by chick liver during de novo lipogenesis [276, 504], and that there may be a differential utilization of palmitic acid, compared with other fatty acids, for neutral lipid synthesis [351].

Some of the forementioned differences between observations from oestrogen-treated immature birds and laying hens are undoubtedly a result of the egg-laying process. In the laying hen, lipid is continually being removed from the body by deposition in the developing oocyte and the laying of eggs. This results in a continual demand for lipid, which is not manifested in the immature bird and cockerel after oestrogen treatment. In these latter cases, accumulation of lipid and egg yolk proteins in the liver cells and blood might be expected to exert feedback inhibition on their hepatic syntheses. For this reason, the events that occur at short intervals after oestrogen treatment of immature birds and cockerels are more likely to be indicative of the situation existing in the laying hen.

However, the differences between oestrogen-treated birds and laying hens outlined above are considered to be relatively minor, and the administration of oestrogen to immature male and female birds and cockerels remains a useful model system for studying the major molecular events involved in avian vitellogenesis. The investigations undertaken in the present study were designed to establish changes in hepatic lipogenesis in the male chick at relatively short intervals ($\leq$ 2 days) after oestrogen treatment.

2. The use of liver slices to measure hepatic lipogenesis in vitro

The use of tissue slices for studying metabolism has several disadvantages, many of which have been outlined in Chapter 3. Major difficulties associated with this preparation are the problems of diffusion of oxygen and substrates into the cells, and the presence of cut cells on the surface and 'shocked' cells in the interior of the slices. In connection with these problems, the use of isolated, perfused livers would be advantageous, since cells do not suffer mechanical damage, and diffusion problems are avoided. However, the adoption of this technique in the present study would have severely limited the number of experiments performed and, therefore, the range of experimental conditions investigated.

Isolated cells are often the system of choice for investigations such as those carried out in the present study. Pilot experiments in this laboratory, using a non-perfusion technique for cell isolation, produced very low yields of cells (approx. 7×10^6 cells/g liver) with rather low viability (approx. 70%) from livers of oestrogen-treated birds. Presumably this was due to the fragility of liver cells in these animals, and the low yield of viable cells made the technique unsuitable for this work. Consequently, tissue slices were considered to be the best whole cell preparation available under the circumstances.

The fragility of cells from oestrogen-treated birds may have resulted in a higher degree of cell damage on preparation of liver slices compared with that obtained for control liver slices, thus giving an artificially low rate of incorporation of precursors into lipids. Therefore, any stimulation of lipogenesis observed in liver slices from oestrogen-treated birds should be regarded as a minimum level.

3. Summary of results obtained in the present study

The main features of the response of the male chick to oestrogen treatment, that resulted from the present study, are summarized below:-

(a) Liver weight

Liver weight increased with time after a single injection of 17β-oestradiol (1 mg/100 g body wt.) up to 50 hours. Liver weights at about 50 hours after oestrogen treatment were up to 2·4-fold greater than those of untreated chicks and control chicks injected with propane-1,2-diol only.

A similar time-related increase in liver weight was observed up to 40 hours after a single injection of 0·75 mg 17β-oestradiol/100 g body weight.

A dose-related increase in liver weight was observed 48 hours after a single injection of 17β-oestradiol over the dose range 0 - 1·25 mg/100 g body weight.

(b) DNA content of liver

Coincident with the increase in liver weight after oestrogen treatment (1 mg 17β-oestradiol/100 g body wt.) was a decrease in the DNA content of a unit weight of liver.

(c) Plasma triacylglycerol concentration

Plasma triacylglycerol concentrations of chicks, after a single injection of 1 mg 17β-oestradiol/100 g body weight, became significantly greater than control values at 5 - $7\frac{1}{2}$ hours post-injection ($P < 0{\cdot}05$), and increased progressively up to 50 hours after injection. The highest elevation of plasma triacylglycerol obtained represented a 33-fold increase in concentration compared with control levels.

(d) Plasma phosphoprotein concentration

Plasma phosphoprotein concentrations are indicative of vitellogenin levels, and after treating chicks with a single injection of 1 mg 17β-oestradiol/100 g body weight, the levels of phosphoprotein became significantly greater than control levels at 14 - 18 hours post-injection ($P < 0{\cdot}02$). Values for oestrogen-treated chicks increased progressively up to about $42\frac{1}{2}$ hours, although values remained high up to 55 hours post-injection. The greatest elevation of plasma phosphoprotein concentration represented an 8-fold increase compared with control values.

(e) Liver slice lipogenesis

In the studies described below, oestrogen-treated chicks received a single injection of 17β-oestradiol (1 mg/100 g body wt.) in propane-1,2-diol, and control chicks received an equivalent volume of propane-1,2-diol only.

(i) [1-^{14}C] Acetate incorporation studies

Maximum differences in acetate incorporation into total lipid between oestrogen-treated and control chicks occurred at 17 - $21\frac{3}{4}$ hours after injection. When results were expressed as nmoles acetate incorporated/100 mg liver/hour, values for oestrogen-treated chicks at this time were significantly greater ($P < 0{\cdot}01$) than those for control chicks. This difference between oestrogen-treated and control values at 17 - $21\frac{3}{4}$ hours became greater when the results were expressed on a total liver or a cellular basis. The mean total incorporation for oestrogen-treated chicks at this time was 3-fold greater than that for control chicks.

Significantly greater incorporation values were obtained for oestrogen-treated chicks than for control chicks at $48\frac{3}{4}$ - $55\frac{1}{2}$ hours after injection when results were expressed on a cellular basis ($P < 0{\cdot}05$).

Saponification analyses of lipid extracts from liver slices revealed that the majority of the recovered radioactivity (> 84%) was located in fatty acids derived from complex lipids. The percentage of the recovered radioactivity in non-saponifiable lipids was significantly greater for liver slices from oestrogen-treated chicks than for those from control chicks ($P < 0{\cdot}05$). Analyses of lipid extracts from liver slices by t.l.c. demonstrated that 66·5 - 69·5% of the recovered radioisotope was located in triacylglycerol. A significantly greater percentage of the recovered radioactivity was located in phospholipid in lipid extracts from control chick liver slices, than was obtained for lipid extracts from oestrogen-treated chick liver slices ($P < 0{\cdot}02$).

(ii) $^{3}H_2O$ incorporation studies

Expression of the results for $^{3}H_2O$ incorporation into total lipid on a total liver or a cellular basis, revealed significantly greater values for oestrogen-treated chicks than for control chicks at 14 - 17, 19 - $21\frac{3}{4}$, $25\frac{1}{4}$ - $28\frac{3}{4}$ and $40\frac{1}{4}$ - $43\frac{1}{4}$ hours after injection. The greatest differences between results for oestrogen-treated and control chicks were obtained at $40\frac{1}{4}$ - $43\frac{1}{4}$ hours. The mean total organ incorporation for oestrogen-treated chicks was 2·4-fold greater than that for control chicks at this time.

Expression of the incorporation results as µg atoms H incorporated/100 mg liver/hour, as µg atoms H incorporated/liver/minute or as µg atoms H incorporated/0·1 mg liver DNA/ hour, revealed that values for untreated chicks were significantly lower than the results for control chicks at $3\frac{1}{2}$ - $7\frac{1}{4}$ hours and 14 - 17 hours after injection of propane-1,2-diol.

Saponification analyses of lipid extracts from liver slices revealed that the majority of the recovered radioactivity (> 93%) was located in fatty acids derived from complex lipids. T.l.c. analyses of lipid extracts from slices demonstrated that approximately 87% of the

recovered radioisotope was located in triacylglycerol. There were no significant differences between the results for control and oestrogen-treated liver lipid extracts.

(iii) [9,10-^{3}H] Palmitate incorporation studies

When results of the [9,10-^{3}H] palmitate incorporation studies were expressed as nmoles palmitate incorporated into triacylglycerol/100 mg liver/hour, values for oestrogen-treated chicks at 19 - 22¼, 28 - 31¾ and 40½ - 44 hours after injection were significantly greater than those for control chicks. Expression of the results on a total liver basis or on a cellular basis, revealed significantly greater values for oestrogen-treated chicks than for control chicks at 14½ - 18½, 19 - 22¼, 28 - 31¾ and 40½ - 44 hours after injection.

The greatest differences between oestrogen-treated and control chicks were obtained at 40½ - 44 hours post-injection. The mean total organ incorporation for oestrogen-treated chicks was 2·47-fold greater than that for control chicks at this time.

T.l.c. analyses of lipid extracts from liver slices showed that the majority of the recovered radioactivity (> 92%) was located in triacylglycerol. A significantly greater percentage of the recovered radioactivity was located as free fatty acid in control and untreated chick liver slices than in oestrogen-treated chick liver slices ($P < 0{\cdot}01$). In addition, a significantly greater percentage of the recovered radioactivity was located in triacylglycerol for oestrogen-treated chick liver slices than for liver slices of control chicks ($P < 0{\cdot}01$).

(f) Hepatic fatty acid synthetase activity

Chicks received a single injection of 17β-oestradiol (0·25 - 1·25 mg/100 g body wt.) in propane-1,2-diol or of propane-1,2-diol only, and hepatic fatty acid synthetase activity was assayed 48 hours later. Maximum mean specific activity and mean total organ activity were ob-

served with a dose of 0·75 mg 17β-oestradiol/100 g body weight.

Treatment of chicks with 0·75 mg 17β-oestradiol/100 g body weight yielded results for hepatic fatty acid synthetase activity which suggested that early increases in specific activity and total organ activity (≤ 26 h) are caused by handling and/or injection of propane-1,2-diol, and that oestrogen-dependent increases occur within 48 hours.

In many cases, the mean soluble protein content/g liver was observed to decrease significantly after oestrogen treatment, compared with the levels for untreated and control chick livers.

4. Sequence and correlation of changes in plasma triacylglycerol and phosphoprotein levels, liver slice lipogenesis and hepatic fatty acid synthetase activity

The sequence of the changes in lipid metabolism that occur after oestrogen treatment of the male chick is important if the significance and a full understanding of these events is to be appreciated.

Examination of results obtained in Chapters 3 and 4 indicate that, compared with untreated chicks, the control chicks exhibited:-

(a) significantly greater incorporation of tritium from $^{3}H_2O$ into total lipid of liver slices up to 17 hours post-injection (Chapter 3).

(b) significantly greater specific activities and activities/g liver of hepatic fatty acid synthetase up to 26 hours post-injection (Chapter 4).

The results obtained from these two lines of approach, therefore, indicate that a stimulation of fatty acid synthesis at early times after injection may be caused by handling and/or injection of propane-1,2-diol, and emphasize the need to include suitable control animals in all experiments. The inclusion of control animals at all stages is extremely important, particularly when using young animals for experimen-

tation, since metabolic pathways such as lipogenesis and the activities of associated enzymes are known to be extremely variable during the first few weeks of neonatal life (30, 359, 413).

The results of the liver slice studies and fatty acid synthetase study are not directly comparable, since different hormone doses were administered in each case. In the liver slice studies, oestrogenized chicks were treated with 1 mg 17β-oestradiol/100 g body weight, whilst in the enzyme study they received 0·75 mg 17β-oestradiol/100 g body weight. The responses to oestrogenization may, therefore, differ in time course and magnitude because of this dose difference.

The greatest difference in the rate of lipogenesis between oestrogen-treated and control chicks, as measured by the incorporation of [1-^{14}C] acetate into total lipid, occurred at 17 - 21¾ hours after injection, when the mean total organ incorporation for oestrogen-treated chicks was 3-fold greater than that for control chicks. In comparison, the greatest difference in the rate of lipogenesis between oestrogen-treated and control chicks, assayed by $^{3}H_2O$ incorporation into lipids, occurred at 40¼ - 43¼ hours after injection, when the mean total organ incorporation for oestrogen-treated chicks was 2·4-fold greater than that for control chicks. These stimulations of the rate of lipogenesis are greater than the stimulations obtained in fatty acid synthetase activity after oestrogen treatment of chicks. The greatest difference in total liver fatty acid synthetase activity between oestrogen-treated (0·75 mg 17β-oestradiol/100 g body wt.) and control chicks was observed at 48 hours after injection, when the mean total organ activity for oestrogen-treated chicks was 1·76-fold greater than that for control chicks. The differences in magnitude of these responses may be the result of the differing hormone dose. It would appear, therefore, that an increase in fatty acid synthetase activity could well contribute

to the increases in the rate of lipogenesis observed in oestrogen-treated chicks, compared with control chicks, at times later than 26 hours post-injection. Differences in the rate of lipogenesis between oestrogen-treated and control chicks observed at times earlier than 26 hours after injection would appear to occur independently of an increase in fatty acid synthetase activity, since fatty acid synthetase activity, measured *in vitro* under extracellular 'optimum' conditions, was found to be unaffected by oestrogen until after 26 hours. These earlier changes in fatty acid synthesis could, however, be governed by increased activities of other lipogenic enzymes such as acetyl-CoA carboxylase, and/or by increased substrate availability.

[1-^{14}C] acetate is a relatively poor precursor to use in monitoring *de novo* fatty acid synthesis in chick liver cells, since it is not a highly physiological substrate and also does not define changes in *de novo* lipogenesis as well as $^{3}H_2O$ (Chapter 3). Hence, measurement of the rate of lipogenesis using [1-^{14}C] acetate may be deceptive if there are changes in the pool size of acetyl-CoA, and if there are problems associated with the conversion of acetate to acetyl-CoA in chick liver cells.

The results of the $^{3}H_2O$ and [9,10-^{3}H] palmitate studies revealed that significantly greater total organ incorporation values were obtained for oestrogen-treated chicks than for control chicks from 14 to 44 hours after injection. The greatest difference, in both cases, was in the approximately 40 - 44 h group, and the relative magnitude of stimulation of incorporation was similar in the two instances. A 2·40-fold increase in mean total incorporation was obtained in the $^{3}H_2O$ studies, compared with a 2·47-fold increase in the [9,10-^{3}H] palmitate studies. This suggests that *de novo* fatty acid synthesis

may be as important as complex lipid formation from fatty acids in the hepatic lipogenic response of the chick after oestrogen treatment.

Comparison of the time courses of the increases in plasma triacylglycerol and phosphoprotein concentrations after oestrogen treatment of male chicks, reveals that the increase in plasma triacylglycerol levels became significant at 5 - 7½ hours after injection, which was several hours before a significant increase in phosphoprotein levels became detectable at 14 - 18 hours post-injection. This sequence of changes is in agreement with the report of Bergink et al. (81), in which the rise in concentration of plasma VLDL occurred earlier than the increase in plasma phosphoprotein concentration.

In contrast, a significant oestrogen-dependent stimulation of the rate of de novo hepatic fatty acid synthesis (monitored by $^{3}H_2O$ incorporation into total lipid of liver slices), and of the rate of hepatic glycerolipid synthesis from pre-formed fatty acid (monitored by [9,10-^{3}H] palmitate incorporation into triacylglycerol of liver slices), did not occur until at least 14 - 14½ hours post-injection. However, comparisons of mean incorporation values, expressed on a total organ basis and a cellular basis, indicate that de novo lipogenesis, assayed by measuring the incorporation of [1-^{14}C] acetate or $^{3}H_2O$ into lipids, might be increased slightly during the approximately 3½ - 8 h period following oestrogen treatment and, hence, could contribute to the early increases in plasma triacylglycerol levels. On the other hand, oestrogen-induced increases in fatty acid synthetase activity, as measured in vitro, did not emerge until at least 26 hours after the injection of 17β-oestradiol (0·75 mg/100 g body wt.). This phenomenon of non-parallel and non-coordinate changes in fatty acid synthesis in

whole cells and the activities of lipogenic enzymes has been observed many times in the literature (33, 45, 46, 49, 50, 351, 375, 410, 420, 424).

SUGGESTIONS FOR FURTHER WORK

1. In vitro measurement of hepatic lipogenesis

A major problem associated with the use of [1-^{14}C] acetate to study de novo lipogenesis in liver slices is the need to measure the intracellular acetyl-CoA pool size, since variations would lead to differences in the specific radioactivity of acetyl-CoA. In addition, lipogenesis may be limited by the rate of entry of acetate into the cell, or by its conversion to acetyl-CoA. Hence, measurements of acetyl-CoA pool sizes in the cells of liver slices from oestrogen-treated and control chicks would increase the validity of using [1-^{14}C] acetate to measure de novo lipogenesis. This problem of measuring acetyl-CoA pool sizes was overcome in the present study by the use of $^{3}H_2O$ to assay de novo lipogenesis in liver slices, although the use of this precursor may not be ideal. The increase in cell size and the cell water 'pool' caused by oestrogen treatment might lead to a decrease in the specific radioactivity of water in the cell, and hence to an underestimate of de novo lipogenesis. A pool size problem also occurs with studies of glycerolipid synthesis assayed by measuring the incorporation of [9,10-^{3}H] palmitate into triacylglycerol. Hawkins & Heald (236) showed that the rate of incorporation of palmitate into neutral lipids by chick liver slices increased in a linear fashion with increasing free fatty acid concentration, indicating the importance of determining fatty acid pool sizes in the livers, and blood fatty acid

levels, in order to elucidate the events actually occurring *in vivo*.

Since hepatocytes eliminate many of the problems of diffusion and cell damage associated with liver slices, development of techniques to isolate chick hepatocytes in high yield, and their use in incorporation studies, would be particularly useful in extending the studies that have been carried out with liver slices. Isolated hepatocytes would provide a more flexible and controllable experimental system for the study of metabolic changes associated with oestrogen treatment, since the cells could be carried through into primary cell culture and maintained in the presence or absence of oestrogen. This cell preparation would prove extremely useful in studies of the synthesis and secretion of vitellogenin and VLDL, which could be monitored by immunoprecipitation.

2. *In vivo* measurement of hepatic lipogenesis

A clear picture of the state of lipogenesis in the oestrogen-treated male chick can only be achieved by a combination of *in vivo* and *in vitro* techniques, since *in vitro* studies are obviously artificial. *In vivo* studies that would extend the results obtained in the present investigation would be the simultaneous determinations of the initial rates of incorporation of injected $^{3}H_2O$ and ^{14}C-labelled palmitate into liver lipids at various times after oestrogen treatment. This would give more information about the relative importance of *de novo* lipogenesis and of lipogenesis from pre-formed fatty acids *in vivo*.

A more detailed knowledge of the time course of changes in triacylglycerol and free fatty acid levels in the blood after oestrogen treatment would help in the search for any precursor/product relationship that might exist. In this respect, studies of the lipid metabolism of extra-hepatic sites, such as adipose tissue, after oestrogen

treatment, would clarify the importance of the liver and extra-hepatic sites as sources of fatty acids for hepatic glycerolipid synthesis.

3. Changes in lipogenic enzymes *in vivo* and *in vitro*

Assaying the activity of an enzyme *in vitro*, under extracellular 'optimum' conditions, generally gives an indication of the total catalytic activity of the enzyme rather than the activity that actually exists *in vivo*. Changing concentrations of lipogenic enzymes may not always be involved in fluctuating rates of lipogenesis, since alterations in the flux of metabolites through the biosynthetic pathway, and regulation of the activities of constant amounts of enzymes, may be responsible (276, 393, 465). It is advisable to assay several enzymes in a metabolic pathway, since the activities of some enzymes may be altered, whilst the activities of others might remain unchanged.

The importance of assaying a number of enzymes in a metabolic pathway, and having a detailed knowledge of the relative importance of enzymes in the normal animal, is illustrated by the activity of the pentose phosphate pathway in the fowl. In mammals, the pentose phosphate pathway is important in the provision of NADPH for lipogenesis (393, 416). However, this pathway is relatively unimportant in the liver of the domestic fowl (49, 412 - 416), and because of this, the specific activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase have not been found to be significantly different in the livers of laying hens and cockerels (414). Similarly, the specific activities of these enzymes do not increase in the liver during the first few days after hatching, at a time when hepatic lipogenesis increases enormously (30, 413), and fasting and refeeding do not significantly affect the activities of these enzymes in the liver (49). Consequently, determining the possible lipogenic capacity of the liver

of the domestic fowl on the basis of the activities of the pentose phosphate pathway enzymes would be incorrect.

In the present study, the lack of an oestrogen-induced increase in fatty acid synthetase activity, as measured in vitro, during the first 26 hours after oestrogen injection (0·75 mg 17β-oestradiol/100 g body wt.) indicated that any oestrogen-induced increases in fatty acid synthesis during this time may not be brought about by an increase in fatty acid synthetase activity. However, this does not rule out the possibility that acetyl-CoA carboxylase, another key enzyme in the fatty acid synthetic sequence, may increase in activity during the 26 hours following oestrogen treatment. The activity of hepatic acetyl-CoA carboxylase, after oestrogen treatment of male chicks, is currently being studied in this laboratory. Most hepatic enzyme studies performed at short intervals after oestrogen treatment have been carried out using immature female chickens, and have concentrated on 'malic' enzyme and ATP citrate lyase [166, 256, 363], although increased fatty acid synthetase activity has been demonstrated after longer periods of oestrogen treatment [31]. On the other hand, very few studies of lipogenic enzyme activities have been reported for oestrogen-treated male birds [244, 254, 296, 464].

Any significant changes in enzyme activity resulting from such studies could be further characterized by:-

(a) purification of the enzyme and preparation of a monospecific antiserum.

(b) use of the monospecific antiserum to monitor changes in the amount of enzyme protein, to distinguish between changes in the catalytic efficiency of a fixed amount of enzyme protein and changes in the quantity of enzyme protein.

In addition, the turnover of lipogenic enzymes could be studied in

primary hepatocyte cultures, maintained in the presence or absence of oestradiol. The rates of synthesis of specific lipogenic enzymes could be quantified, after incubation of the cells with isotopically-labelled amino acids, by disruption of the cells and isolation of the specific lipogenic enzymes by immunoadsorption techniques. The rates of degradation of specific enzymes could also be quantified in a similar way. After incubation of the cells with an isotopically-labelled amino acid, cells could be placed into culture medium containing non-radioactive amino acids, and at various times during this 'chase' period specific isotopically-labelled lipogenic enzymes could be isolated by immunoadsorption and quantified.

In the present study, the results for hepatic fatty acid synthetase activity suggested that early increases in activity ($\leq$ 26 h) were caused by handling and/or the injection of propane-1,2-diol. However, the experiments do not allow us to distinguish between a possible stress effect caused by the acts of handling and injection, and a possible effect of propane-1,2-diol on enzyme activity. The inclusion of further control chicks, injected with saline instead of propane-1,2-diol, would allow us to distinguish between these alternative explanations for the increases in enzyme activity up to 26 hours after injection. This procedure is currently being adopted in this laboratory, during studies of hepatic acetyl-CoA carboxylase activity after oestrogen treatment of male chicks.

4. Effects of food intake

In the present study, birds received food and water ad libitum until death, in the light of the observations of Yeh & Leveille [45], who showed that hepatic fatty acid synthesis was depressed after fasting chicks for short periods. Thirty minutes after food had been withdrawn

fatty acid synthesis was reduced, and the rate of fatty acid synthesis was reduced to about 10% of the rate in ad libitum-fed chicks after fasting for 2 hours. The presence of the crop as a food storage organ in the chick prevents the imposition of uniform food deprivation and, therefore, it was considered important to ensure that all the chicks were well-fed. However, several workers have demonstrated that oestrogen-treated birds show an enhanced food intake (174, 246). This is contrary to the situation in mammals, in which reduced feeding has been shown to occur around ovulation and following oestrogen treatment (505 - 508). Lepkovsky & Furuta (270) demonstrated that hyperphagia leads to enhanced hepatic lipogenesis in White Leghorn cockerels. Similarly, Balnave (456) indicated that equalized food and water intakes prevent differences in chick hepatic lipid metabolism, caused by thyroxine treatment, being obscured by the effects of differing intakes. Therefore, the adoption of equalized food and water intakes in studies of oestrogen-treated and control chicks might clarify differences in hepatic lipid metabolism that are independent of food consumption.

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APPENDIX

COMPOSITION OF THE CHICK DIET

404 Gold-Start Crumbs/ACS (Dalgety Agriculture Ltd., Dalgety House, Clifton, Bristol 8.)

Oil	3%
Fibre	4%
Ash	7%
Protein	18%

Permitted antioxidant

Vitamin A (i.u./kg)	10000
Vitamin D3 (i.u./kg)	2000
Vitamin E (i.u./kg)	15
Molybdenum (mg/kg)	2
Selenium (mg/kg)	0·30

Anti-coccidial supplement: Pancoxin (PL0025/4010):-

100 mg/kg Amprolium

60 mg/kg Sulphaquinoxaline

5 mg/kg Ethopabate